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Characterization of the immune responses to cancer associated antigens is considered to be important in the development of new therapeutic strategies for the treatment of breast cancer (BC). Recent advances in our basic understanding of antigen recognition by T lymphocytes has led to the definition of several human tumor-associated antigens (TAA) recognized by cytotoxic T lymphocytes (CTLs). However, it has been noted that the expression of these TAAs is relatively low in BC tumor cells. A new protein named mammaglobin has been demonstrated to be exclusively expressed in the mammary epithelium. In addition, 90% of primary BC tumors have detectable levels of mammaglobin protein. Given the exclusive mammaglobin expression in BC tumors, this novel protein may prove to be a highly specific TAA that could be utilized in the near future for *in vitro* BC-specific activation of CTLs. During the award period, we have demonstrated that mammaglobin is antigenic and breast cancer patients have T cells in their circulation capable of recognizing this protein. Further, we have identified mammaglobin-derived antigenic peptides that are highly expressed in BC tumor tissue and are recognized by human CTLs. These findings offer many exciting avenues both towards its use for prognostic purposes as well as developing new therapeutic options for the treatment of BC.

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INTRODUCTION

The elucidation of the immune response to cancer should be of great help in the development of new therapeutic strategies for the treatment of breast cancer (BC), which is the second leading cause of cancer death among American women. A new alternative is adoptive immunotherapy which is based on the observation that CD8+ cytotoxic T lymphocytes (CTL) may be generated both *in vivo* and *in vitro* with the ability to lyse autologous tumor cells. However, attempts at active specific immunotherapy using cancer vaccines have met with little success in clinical trials. Based on recent advances in our basic understanding of antigen recognition by T lymphocytes, it has been possible to identify several human tumor-associated antigens (TAA) recognized by CTLs. These TAAs are expressed in a wide variety of tumor types including BC, however, the expression for these TAAs has been shown to be relatively low in BC tumor cells.

A new gene named mammaglobin has been identified in our laboratory. Mammaglobin mRNA has been demonstrated to be exclusively expressed in the mammary epithelium as compared to 15 different normal and fetal tissues tested. Interestingly, mammaglobin is only expressed during mammary gland proliferation such as occurs during pregnancy and lactation. In addition, 90% of primary BC tumors have detectable levels of mammaglobin protein expression. We have also shown that 60% of peripheral blood stem cell (PBSC) collections from patients undergoing high dose chemotherapy for metastatic BC were positive for mammaglobin mRNA. Given the high frequency of mammaglobin expression in BC tumors, its relative low expression in normal breast tissue, and its total lack of expression in other normal tissues, this novel protein may prove to be a highly specific BC-associated antigen that could be utilized in the near future for *in vitro* BC-specific activation of CTLs. Mammaglobin-specific CTLs would be of immediate benefit in selectively removing BC tumor cells from PBSC or bone marrow collections prior to autologous transplantation in BC patients undergoing high dose chemotherapy. The discovery of mammaglobin-derived antigenic peptides that are highly expressed in BC tumor tissue and are recognized by CTLs offer many exciting future therapeutic options for the treatment of BC for which there are currently no effective systemic therapies for advanced disease. The goals of this study are: 1) to determine whether CD4+ and CD8+ T cell-mediated immune responses generated *in vivo* in BC patients to their tumors (tumor-infiltrating lymphocytes) can recognize mammaglobin-derived peptides, 2) to determine whether mammaglobin-reactive CTLs generated *in vitro* have the ability to lyse BC tumor cells, 3) to identify the mammaglobin epitopes recognized by CTLs presented by widely expressed HLA class I molecules, and 4) to determine the potential use of mammaglobin cDNA vaccination in the generation of a BC-specific CTL immune response.

BODY

Task 1. To determine whether breast cancer-specific T cell immune responses generated *in vivo* can recognize mammaglobin-derived antigenic peptides.

Lack of mammaglobin-specific T cell proliferative responses in healthy female individuals as compared to healthy male individuals. The expression of mammaglobin has been shown to be restricted to the normal adult mammary epithelium and is overexpressed in 23% of primary breast tumors. Therefore, normal individuals would show specific T cell unresponsiveness (tolerance) against mammaglobin. However, the possibility exists that the tissue-specific mammaglobin expression may be regulated by steroid hormones as is the expression of all the uteroglobin family member. This hormone-regulated expression of mammaglobin may lead to a differential expression of mammaglobin in the adult mammary epithelium between healthy female and male individuals and hence to differential levels of T cell responsiveness to this same autoantigen. To test this, we produced recombinant mammaglobin by subcloning the mammaglobin cDNA into a PCI-neo expression vector and consequent transformation of *E. coli*. Then, we compared the T cell proliferative responses to recombinant mammaglobin between healthy adult female and male individuals as described (**Appendix 4**). As observed in **Figure 1 (Appendix 5)**, peripheral blood mononuclear cells (PBMC) derived from healthy adult male individuals showed significantly higher levels of proliferation to mammaglobin as compared to PBMCs from healthy female individuals. Interestingly, no

proliferative responses were observed in healthy adult males or females individuals to another autoantigen, human albumin, which is expressed in comparable levels in individuals from both sexes. These data indicate that adult female individuals are tolerant to mammaglobin. Therefore, mammaglobin-specific T cells in healthy adult females may be absent from the periphery (clonal deletion) or display antigen-specific unresponsiveness against mammaglobin (anergy).

Higher frequency of mammaglobin-reactive T cells in breast cancer patients than in healthy female individuals. To address the question whether mammaglobin-specific T cells are deleted or anergized in adult females, we determined the precursor frequency of mammaglobin reactive T cells in healthy adult female individuals (controls) and breast cancer patients. Briefly, we determined the frequency of mammaglobin-reactive CD4⁺ T helper cells and CD8⁺ cytotoxic T cells using autologous dendritic cells pre-pulsed with recombinant mammaglobin (**Appendix 4**). If the mammaglobin-specific T cells are anergic, the possibility exists that selective mammaglobin-specific tolerant T cells clones may respond to this protein in case of aberrantly high expression of the protein as observed in breast tumors. In this case, we would observe a higher frequency of mammaglobin-reactive T cells in a selected group of breast cancer patients. On the other hand, if mammaglobin-specific T cell clones had been deleted we would not be able to observe a higher frequency of mammaglobin-reactive T cells in breast cancer patients. As shown in **Table 2 (Appendix 5)**, the frequency of mammaglobin-specific proliferating T cells in peripheral blood of breast cancer patient ($3.5 \times 10^{-5} \pm 3.2 \times 10^{-5}$) were significantly higher than that observed in healthy female control individuals ($0.15 \times 10^{-5} \pm 0.17 \times 10^{-6}$) ($P=0.03$, Student's T tests). Only one of the seven patients included in this study did not respond to recombinant mammaglobin (patient No. 7).

In addition, the frequency of mammaglobin-reactive CD8⁺ CTLs in peripheral blood of breast cancer patients ($2.4 \times 10^{-5} \pm 1.8 \times 10^{-5}$) was also significantly higher than that observed in healthy female control individuals ($0.39 \times 10^{-5} \pm 0.52 \times 10^{-5}$) ($P=0.03$, Student's T test) (**Table 2, Appendix 5**). Interestingly, one of the seven healthy female control individuals included in this study showed a particularly high CTL precursor frequency (control No. 4).

The results presented herein indicate that, even though healthy female individuals do not respond to mammaglobin as a normal auto-antigen, they have mammaglobin-specific T cell clones that expand *in vivo* as result of an aberrant over-expression of this protein in breast cancer cells.

Production of tumors in severe combined immunodeficient mice. In order to develop methods to grow human tumors in SCID mice, we initiated our studies with a human breast cancer cell line. The HBL-100 cells were grown in Severe Combined Immunodeficient (SCID) mice (Jackson laboratory, Bar Harbor, ME) by injecting 2.5×10^6 cells in 0.5 ml of Hank buffer solution (Gibco BRL, Grand Island, NY) subcutaneously into the flanks of the mice. Tumors were allowed to grow to 4-5cm in diameter then excised and 10g was used for peptide isolation. A section of the tumor was fixed in 10% formalin (Sigma, St. Louis, MO) and paraffin embedded. Paraffin-embedded tissues were sectioned at 5 μ m, attached to glass slides, and deparaffinized as described by Saarialho-Kere et al (1). Endogenous peroxidase activity was blocked by incubating the slides in 0.3% H₂O₂ for 30 minutes at room temperature. Mouse-anti-human vimentin monoclonal antibody (Biogenex, San Ramon, CA) was used at 1:10,000 dilution. The cytokeratin antibody was a cocktail of mouse-anti-human cytokeratin monoclonal antibodies including MAK6 (Zymed Laboratories Inc., South San Francisco, CA) at 1:20 dilution, cytokeratin AE1/AE3 (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 1:150 dilution, and cytokeratin CAM 5.2 (Becton Dickinson, Mountain View, CA) at 1:150 dilution. All immunohistochemical staining was done following microwave antigen retrieval in 0.1M citrate buffer. The primary antibodies were incubated overnight at 4°C. The secondary antibody, a biotinylated horse-anti-mouse IgG (Vector Laboratory, Burlingame, CA), was used at 1:200 dilution. Immune complexes were detected using a Vectastain ABC Elite kit (Vector Laboratory, Burlingame, CA) with diaminobenzidine tetra hydrochloride (DAB) as the chromogenic substrate. Slides

were counter stained with Harris hematoxylin (Sigma, St. Louis, MO) and examined with a Nikon microscope (Nikon, Garden City, NY). McCoy's medium (Sigma, St. Louis, MO) was used as the control. Staining of the SCID mouse intestinal epithelial cells were done for comparison. Hematoxylin and eosin staining were also done.

HBL-100 tissues harvested from SCID mice are of human origin. To ascertain that the cancer tissues which were harvested from SCID mice are of human origin, immunohistochemical staining of the tissues with mouse-anti-human vimentin and keratin was performed and compared to staining of SCID mouse intestinal sections (negative control). Cell morphology was examined in hematoxylin and eosin stains of the tumor sections which showed the cells to have round to oval nuclei, vesicular chromatin, eosinophilic centrally placed nucleoli, moderate pleomorphism, moderately abundant cytoplasm, and frequent mitotic figures consistent with poorly differentiated carcinoma. Immunohistochemical staining using mouse-anti-human vimentin and keratin antibodies confirmed the human origin of the tumor (**Appendix 1**).

Similar results were also obtained with other breast cancer tumor cell lines including MCF-7 and AU565 which both express HLA-A2 on their cell surface.

Task 2. To determine whether mammaglobin-reactive CD8+ CTLs generated *in vitro* have the ability to lyse breast cancer cells.

Mammaglobin-specific proliferative response by CD8+ CTL lines generated *in vitro*. Based on the observation that mammaglobin-specific CTLs are normally expanded in breast cancer patients *in vivo*, the possibility existed that high affinity mammaglobin-specific CTL lines could be generated *in vitro*. To test this, we generated a mammaglobin-specific CD8+ T cell line from a HLA-A2-positive, breast cancer patient by means of five weekly stimulations with dendritic cells pre-pulsed with recombinant mammaglobin or two different HLA-A2-binding, mammaglobin-derived peptides (SKM-67: KLLMVLMMLA [aminoacids 2-10] and SKM-68: LLMVLMMLAA [aminoacids 3-11]) (**Appendix 4**). The HLA-A2-binding capabilities of these mammaglobin-derived peptides were predicted by means of the Bioinformatics and Molecular Analysis Section Program (www.bimas.dcrf.nih.gov/molbio/hla_bind/). The SKM-67 and SKM-68 peptides were used in these studies because they presented the highest affinities (longer complex disassociation times) for the HLA-A2 molecule. As shown in **Figure 2 (Appendix 5)**, the resulting CD8+ CTL line produced against recombinant mammaglobin showed a significantly high proliferative response in the presence of recombinant mammaglobin but not in the presence of bovine serum albumin (control 1) or human albumin (control 2). This data indicate the mammaglobin-specificity of this reaction.

In parallel experiments, the peptide-specific proliferation of the CD8+ CTL lines generated against the SKM-67 and SKM-68 peptides was also determined. As shown in **Figures 3A and 3B (Appendix 5)**, both of these CD8+ CTL lines demonstrated peptide-specific proliferation upon stimulation with cognate peptide, respectively. Since both peptides shared the common anchor residues (L in position 2 and A in position 9), proliferation was observed in reciprocal combination, although the responses were weaker as compared to the corresponding cognate peptide.

Mammaglobin-specific cytotoxic activity by CD8+ T cell lines generated *in vitro*. The results shown above indicate the specific reactivity against mammaglobin of three different CD8+ CTL lines generated *in vitro*. To analyze the mammaglobin-specific CTL activity of the CD8+ T cell line generated against recombinant mammaglobin, we determined its ability to lyse autologous dendritic cells pulsed with recombinant mammaglobin. As shown in **Figure 4 (Appendix 5)**, this particular CD8+ CTL line demonstrated antigen-specific killing of recombinant mammaglobin-pulsed autologous dendritic cells. No lytic activity was observed with non-pulsed autologous dendritic cells (control 1) as well as autologous dendritic cells pulsed with human albumin (control 2). The lytic activity observed with the mammaglobin-pulsed dendritic cells was significantly inhibited by pre-treatment with the W6/32 anti-HLA-class I

monoclonal antibody but not with the KU1A-2 anti-HLA-DR monoclonal antibody. These data demonstrate the HLA class I-restricted recognition of mammaglobin-derived epitopes by this CD8+ CTL line.

In addition, as shown in **Figure 5A and 5B (Appendix 5)**, CD8+ CTL lines obtained *in vitro* against the SKM-67 and SKM-68 peptides also show a significant CTL activity against T2 cells pulsed with the corresponding cognate peptide. This result indicates that both SKM-67 and SKM-68 are recognized in the context of HLA-A2 the CTL lines generated *in vitro*.

Mammaglobin-specific MHC-restricted cytotoxic activity on breast cancer cells by CD8+ T cells generated *in vitro*. The CD8+ CTL line generated *in vitro* against autologous dendritic cells pre-pulsed with recombinant mammaglobin was tested for its ability to lyse several breast cancer cell lines that naturally express the mammaglobin protein (**Table 1, Appendix 5**). As shown in **Figure 6 (Appendix 5)**, this CD8+ CTL line selectively killed mammaglobin-positive breast cancer cell lines in the context of HLA-A2 (HBL-100 and AU565). No killing was observed with mammaglobin-positive, HLA-A2-negative breast cancer cell lines (MDA-MB-415 and MDA-MB-361) or mammaglobin-negative, HLA-A2-positive breast cancer cell lines (MCF-7 and MDA-MB-231). These results clearly demonstrate the MHC restriction and antigen specificity of the CTL activity mediated by mammaglobin-specific CD8+ CTLs generated *in vitro*.

Task 3. To determine the mammaglobin CD8+ T cells epitopes presented by HLA-1, HLA-A2 and HLA-A3.

Mammaglobin-specific, HLA-A3-restricted, cytotoxic activity by CD8+ T cells generated *in vitro* is directed against one dominant antigenic peptide. In order to identify the dominant mammaglobin-derived epitope(s) presented in the context of the HLA-A3 molecule, we developed two CD8+ T cell lines from one HLA-A3 individual by means of culture in the presence of HLA-A3-transfected T2 cells (T2-HLA-A3+) pre-pulsed with eight HLA-A3-binding, mammaglobin-derived peptides (MG-2: FLNQTDDEL, MG-3: LMVLMAL, MG-5: KLLMVLMLA, MG-6: TTNAIDELK, MG-7: KTINPQVSK, MG-9: PLEENVISK, MG-11: LMLAALSQH, and MG-12: AIDELKECF). The prediction of these epitopes was made using the Bioinformatics and Molecular Analysis Section program (www.bimas.dcrf.nih.gov/molbio/hla_bind/). These peptides were used in these studies because they presented the highest affinities for the HLA-A3 molecule. Briefly, After monocyte depletion, peripheral blood mononuclear cells were cultured in presence of irradiated, peptide-loaded T2-HLA-A3+ cells in the presence of recombinant IL-2. After five weekly stimulations, a standard ⁵¹Cr release assay was performed using T2-HLA-A3+ cells pulsed with the mammaglobin-derived peptides as targets. As shown in **Figure 7 (Appendix 5)**, significant cytotoxic activity was observed only against the T2-HLA-A3+ cells loaded with the peptide PLEENVISK (MG-9, aminoacids 23-31). No significant killing was observed against T2-HLA-A3 cells loaded with the other mammaglobin-derived peptides. These results clearly indicate that even though this CD8+ T cell line was developed against several HLA-A3-binding peptides, only the MG-9 peptide is able to stimulate the development of CD8+ CTLs. Similar experiments are currently being performed in our laboratory to determine the dominant mammaglobin epitopes presented in the context of HLA-A2 molecules.

In order to determine whether the CD8+ CTL line directed against the MG-9 peptide had the ability to lyse HLA-A3-positive breast cancer cells that express mammaglobin, we performed a CTL activity assay using as targets several breast cancer cell lines (**Table 3, Appendix 5**). As shown in **Figure 8 (Appendix 5)**, the anti-MG-9 CTL line showed significant cytotoxic activity against one mammaglobin-positive, HLA-A3-positive breast cancer cell line (DU4475). No killing was observed in K-562 cells which indicates the lack of NK cell activity. No CTL activity was observed against mammaglobin-negative, HLA-A3-positive breast cancer cells (T-47D), mammaglobin-positive, HLA-A3-negative breast cancer cells (HBL-100), and mammaglobin-negative, HLA-A3-negative breast cancer cells (MCF-7).

The results presented herein clearly indicate that anti-mammaglobin CD8+ T cell lines developed *in vitro*

have the ability to kill breast cancer cells in a MHC-restricted manner and that the MG-9 peptide is a naturally produced mammaglobin epitope presented by HLA-A3 breast cancer cells. Interestingly, no CTL activity was observed against one mammaglobin-positive, HLA-A3-positive breast cancer cell line (AU-565). However, flow cytometric analysis performed to determine the levels of expression of the HLA-A3 gene in this cell line showed no HLA-A3 expression as compared to the DU4475 cell line. This result further indicates that the MG-9 peptide is naturally expressed in the context of the HLA-A3 molecule.

Epitope immunodominance of the anti-mammaglobin CD8+ CTL response developed *in vivo* in breast cancer patients. As shown above, we observed that breast cancer patients developed a detectable CD8+ CTL response to mammaglobin *in vivo*. To identify the mammaglobin-derived determinant(s) recognized by CD8+ CTLs in breast cancer patients, we have performed anti-IFN- γ ELISPOT assays in five HLA-A3 breast cancer patients and five HLA-A2 breast cancer patients. Toward this, we have stimulated PBMCs from these patients with the eight HLA-A3-binding, mammaglobin-derived peptides (see above) or seven HLA-A2-binding, mammaglobin derived peptides (MG-1: TLSNVEVFM, MG-2: FLNQTDETL, MG-3: LMVLMMLAAL, MG-4: TINPQVSKT, MG-5: KLLMVLMMLA, MG-8: LIYDSSLCDL, and MG-10: MQLIYDSSL). As mentioned above, the prediction of the HLA-A2 epitopes was made using the Bioinformatics and Molecular Analysis Section program (www.bimas.dcrf.nih.gov/molbio/hla_bind/). These peptides were used in these studies because they presented the highest affinities for the HLA-A2 molecule. Briefly, 2×10^5 PBMCs were cultured in the presence of each individual peptide in 96-well plates. After 48 hours, the frequency of IFN- γ -producing cells was determined by means of a standard ELISPOT assay. As observed on **Figure 9 (Appendix 5)**, results with the HLA-A2 breast cancer patients show that they have a significant reactivity against the peptides FLNQTDETL (MG-2, aminoacids 66-74) and LIYDSSLCDL (MG-8, aminoacids 83-92). In addition, as observed in **Figure 10 (Appendix 5)**, results with the HLA-A3 breast cancer patients show that they have a significant reactivity to the peptides KLLMVLMMLA (MG-5, aminoacids 2-10) and TTNAIDELK (MG-6, aminoacids 55-63). Additional ELISPOT assays are currently being performed in our laboratory to expand the number of patients and peptides in these analyses.

The results presented herein clearly identify the dominant mammaglobin-derived epitopes recognized in the context of HLA-A2 and HLA-A3 molecules by CD8+ CTLs *in vivo*. Future experiments will be performed to determine whether HLA-A2 and HLA-A3 CD8+ CTL lines developed *in vitro* against these particular peptides will have the ability to lyse HLA-matched, mammaglobin-positive breast cancer cells *in vitro*. It is interesting that the HLA-A3 CD8+ CTL line generated *in vitro* against the pool of mammaglobin-derived peptides presented a different epitope dominance (**Figure 7, Appendix 5**) as compared to the dominance observed *in vivo* (**Figure 10, Appendix 5**). This difference in the results may be explained by the fact that the antigen presenting cells in our *in vitro* experiments were T2-HLA-A3+ cells and not natural antigen presenting cells such as macrophages and dendritic cells. It remains possible that peptide determinants that are cryptic when presented by one type of antigen presenting cell become immunodominant when presented by other. However, it is noteworthy that the anti-mammaglobin CD8+ CTL line generated *in vitro* showed a highly significant cytotoxic activity against breast cancer cells (**Figure 8, Appendix 5**).

Task 4. Vaccination with full length mammaglobin-A cDNA induces a CD8+ CTL response to breast cancer cells. To determine the feasibility of mammaglobin-A to be used as a breast cancer vaccine, we constructed a DNA expression vector by encoding the full-length mammaglobin-A cDNA and evaluated the efficacy of intramuscular injection of this plasmid to induce mammaglobin-A-specific immune responses in HLA-A2/human CD8 double-transgenic (HLA-A2+/hCD8+) mice (2). Spleen cells obtained from immunized mice were analyzed for their ability to recognize mammaglobin-A-derived HLA-A2-binding epitopes. By means of an IFN- γ ELISPOT analysis we determined that spleen cells from immunized mice

recognized the Mam-A2.1, Mam-A2.2, Mam-A2.4, and Mam-A2.6 (80-89, MQLIYDSSL) peptides. These results indicated that HLA-A2+ mice recognized a similar epitope pattern as HLA-A2+ breast cancer patients and that this represents a valuable unique animal model to characterize the HLA-A2-restricted CD8+ CTL immune response to mammaglobin-A *in vivo*. Subsequently, we determined whether spleen CD8+ CTLs from immunized mice were able to lyse breast cancer cells in an HLA-A2-restricted and mammaglobin-A-specific manner. As shown on **figure 11 (Appendix 5)**, CD8+ CTLs from immunized mice showed significant cytotoxic activity against the AU-565 and HBL-100 breast cancer cell lines (HLA-A2+/mammaglobin-A+) but not against the DU-4475 (HLA-A2-/mammaglobin-A+) and MCF-7 (HLA-A2+/mammaglobin-A-) breast cancer cell lines. These results also indicate that mammaglobin-A cDNA vaccination could represent a novel approach for the design of effective immunotherapy for breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated that breast cancer patients have higher frequency of mammaglobin-reactive proliferating CD4+ T cells as well as CD8+ CTLs in peripheral blood as compared to normal female individuals.

We have identified that the mammaglobin epitopes recognized by CD8+ CTLs *in vivo* in the context of the HLA-A2 molecule are the peptides FLNQTDETL (aminoacids 66-74) and LIYDSSLCDL (aminoacids 83-92).

We have identified that the mammaglobin epitopes recognized by CD8+ CTLs *in vivo* in the context of the HLA-A3 molecule are the peptides KLLMVLMLA (aminoacids 2-10) and TTNAIDELK (aminoacids 55-63).

We have demonstrated that anti-mammaglobin CD8+ T cell lines developed *in vitro* by means of different protocols display MHC-restricted, mammaglobin-specific cytotoxic activity against breast cancer cells.

We have identified that the mammaglobin epitope recognized by CD8+ CTLs generated *in vitro* in the context of the HLA-A3 molecule is the peptide PLEENVISK (aminoacids 23-31).

Vaccination with full length mammaglobin-A cDNA induces a CD8+ CTL response to breast cancer cells. To determine the feasibility of mammaglobin-A to be used as a breast cancer vaccine, we constructed a DNA expression vector by encoding the full-length mammaglobin-A cDNA and evaluated the efficacy of intramuscular injection of this plasmid to induce mammaglobin-A-specific immune responses in HLA-A2/human CD8 double-transgenic (HLA-A2+/hCD8+) mice (2). Spleen cells obtained from immunized mice were analyzed for their ability to recognize mammaglobin-A-derived HLA-A2-binding epitopes. By means of an IFN- γ ELISPOT analysis we determined that spleen cells from immunized mice recognized the Mam-A2.1, Mam-A2.2, Mam-A2.4, and Mam-A2.6 (80-89, MQLIYDSSL) peptides. These results indicated that HLA-A2+ mice recognized a similar epitope pattern as HLA-A2+ breast cancer patients and that this represents a valuable unique animal model to characterize the HLA-A2-restricted CD8+ CTL immune response to mammaglobin-A *in vivo*. Subsequently, we determined whether spleen CD8+ CTLs from immunized mice were able to lyse breast cancer cells in an HLA-A2-restricted and mammaglobin-A-specific manner. As shown on **figure 11 (Appendix 5)**, CD8+ CTLs from immunized mice showed significant cytotoxic activity against the AU-565 and HBL-100 breast cancer cell lines (HLA-A2+/mammaglobin-A+) but not against the DU-4475 (HLA-A2-/mammaglobin-A+) and MCF-7 (HLA-A2+/mammaglobin-A-) breast cancer cell lines. These results also indicate that mammaglobin-A cDNA vaccination could represent a novel approach for the design of effective immunotherapy for breast cancer.

REPORTABLE OUTCOMES

Publications

1. Nguyen T, Naziruddin B, Dintzis S, Doherty, GM, and **Mohanakumar T**. Recognition of breast cancer-associated peptides by tumor-specific, HLA-class I restricted allogeneic cytotoxic T lymphocytes. *International J of Cancer* 81:4:607-615, 1999.
2. Manna PP and Mohanakumar T. Human dendritic cell mediated cytotoxicity against breast carcinoma cells in vitro. *J Leukocyte Biology* (in press).
3. Jaramillo A, Majumder K, Manna P, Fleming T, Doherty G, DiPersio J, and **Mohanakumar T**. Identification of HLA-A3-restricted CD8+ T cell epitopes derived from mammaglobin-A, a tumor-associated antigen of human breast cancer. *Int J Cancer* (in press).
4. Manna P, Jaramillo A, Majumder K, Fleming T, Doherty G, DiPersio J, and **Mohanakumar T**. Generation of CD8+ cytotoxic T lymphocytes against breast cancer cells by stimulation with mammaglobin-A-pulsed dendritic cells. *Breast Cancer Research Treatment* (submitted).

Abstracts

1. Jaramillo A, Majumder K, Fleming TP, Doherty G, Dipersio J and **Mohanakumar T**. Identification of HLA-A2-restricted T cell epitopes from mammaglobin-A, a new breast cancer-specific antigen. Poster presentation at the ERA of Hope DOD BCRP Meeting, Orlando, September 2002.
2. Jaramillo A, Majumder K, Manna PP, Fleming TP, Doherty G, Dipersio J, and **Mohanakumar T**. Identification of HLA-A3-restricted T cell epitopes from mammaglobin-A, a new breast cancer-specific antigen. Poster presentation at the ERA of Hope DOD BCRP Meeting, Orlando, September 2002.
3. Manna PP and **Mohanakumar T**. Cytotoxicity of breast cancer cells mediated by human dendritic cells. Poster presentation at the ERA of Hope DOD BCRP Meeting, Orlando, September 2002.
4. Jaramillo A and **Mohanakumar T**. Induction of CD8+ T cell cytotoxicity to human breast carcinoma by DNA vaccination with the mammaglobin-A gene in HLA-A2+ mice. Poster presentation at the ERA of Hope DOD BCRP Meeting, Orlando, September 2002.

Personnel Paid from this award:

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CONCLUSIONS

A novel breast cancer-associated antigen, mammaglobin-A, has recently been identified in our laboratory. Unlike other genes expressed by breast tumors, the over-expression of mammaglobin-A is breast cancer-specific. Another significant finding is that 80% of primary breast tumors have high levels of mammaglobin-A expression. Our recent studies have shown that mammaglobin-A-reactive T cells are expanded in breast cancer patients. Also, we were able to identify several mammaglobin-A-derived epitopes presented in the context of HLA-A2 and HLA-A3 molecules. Further, a CD8+ cytotoxic T lymphocyte (CTL) line generated against these epitopes had the ability to lyse mammaglobin-A+ breast cancer cell lines. In addition, HLA-A2+/human CD8+ mice vaccinated with mammaglobin-A cDNA recognized a similar epitope pattern as HLA-A2+ breast cancer patients and showed a significant CD8+ CTL activity against HLA-A2+ human breast cancer cell lines.

Future studies planned based upon the above results will test these hypotheses: 1) Lack of recurrence of breast cancer after appropriate surgical and adjuvant therapy, correlates with optimal CD8+ CTL responses to selected mammaglobin-A derived epitopes, and 2) Vaccination of transgenic mice with either full length cDNA or mammaglobin-A-epitope-MHC class I cDNA constructs will induce a CD8+ CTL

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immune response capable of inducing regression of established tumors. The results obtained from this study should provide basis for developing strategies for vaccination against breast cancer recurrence in high risk patients who have undergone surgical and or adjuvant therapy.

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APPENDICES

Appendix 1 – *International J Cancer* 81:4:607-615, 1999, Nguyen, et.al.

Appendix 2 – *J Leukocyte Biology* 2002 (in press), Manna, et.al.

Appendix 3 – *International J Cancer* 2002 (in press), Jaramillo, et.al.

Appendix 4 – *Breast Cancer Research Treatment* 2002 (submitted), Manna, et.al.

Appendix 5 – Tables 1-3 and Figures 1-11

Appendix 6 - Abstracts presented at the ERA of Hope DOD BCRP Meeting, Orlando, September 2002



RECOGNITION OF BREAST CANCER-ASSOCIATED PEPTIDES BY TUMOR-REACTIVE, HLA-CLASS I RESTRICTED ALLOGENEIC CYTOTOXIC T LYMPHOCYTES

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Strategies to identify tumor-associated antigens rely on the paradigm that tumor-associated peptides presented in the context of HLA-class I are recognized by the cellular immune system. Approaches to isolate tumor-specific cytotoxic T lymphocytes (CTL) from tumor-infiltrating lymphocytes are difficult because long-term growth of the CTL requires autologous tumor cells and lymphocytes (PBL) as feeder cells. In this study, a CTL line (BL.HBL-100 CTL) was generated from PBL from a normal healthy donor by stimulating with irradiated, HLA-class I partially matched breast cancer cell line HBL-100. Activated T lymphocytes generated expressed TCR α/β ⁺ with a predominant CD8⁺ population after 12 stimulations (98.54% CD8⁺ vs. 0.18% CD4⁺). These CTL lysed HLA-A1⁺, but not HLA-A1⁻, breast cancer cell lines. Moreover, HLA-A1⁺, non breast cancer cell lines were not recognized. The lytic activity of BL.HBL-100 CTL against HLA-A1⁺ breast cancer cell lines was blocked by monoclonal antibodies (MAbs) to HLA-class I and CD8, but not by anti-HLA-class II and CD4. Recognition of HLA-A1⁺ breast cancer cells by the CTL was dependent on peptides associated with HLA-class I since the lysis was inhibited by acid elution of HLA bound peptides. HBL-100 tumors were grown in severe combined immunodeficient (SCID) mice. Immunohistochemical staining of the HBL-100 tissue harvested from SCID mice demonstrated human breast cancer cells. HLA-class I molecules were affinity purified from the HBL-100 harvested from the SCID mice; class I bound peptides were eluted and separated by RP-HPLC. Pooled HPLC peptide fractions were tested for reconstituting antigenic epitopes recognized by the BL.HBL-100 CTL and found to reside within fraction 40. Our results show that a tumor reactive, HLA-class I restricted CTL was produced by stimulating normal PBL against an HLA-class I matched breast cancer cell line. We also provide evidence for a breast cancer-associated, HLA-class I bound peptide antigen(s) that reconstitutes the antigenic epitope(s) recognized by these CTL. *Int. J. Cancer* 81:607–615, 1999.

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Breast cancer represents the most common malignancy in women, carrying a high morbidity and mortality. About 1 in 9 women will develop breast cancer in her life time. Surgery remains the mainstay of therapy for early disease. However, despite advances in chemo/radiation therapy, advanced breast cancer still carries a high mortality rate. Therefore, there is a need for more effective therapies. Immunotherapy (*e.g.*, peptide vaccination, adoptive immunotherapy) is a promising modality of treatment. Early reports on melanoma, colon cancer, and pancreatic cancer immunotherapy have shown encouraging results (Gjertsen *et al.*, 1995; Kang *et al.*, 1997; Maxwell-Armstrong *et al.*, 1998).

Melanoma has served as a paradigm for tumor antigenic peptide studies because of the relative ease of propagating of melanoma cell lines in vitro and generating CTL clones. Several melanoma antigenic peptides, 8–10 amino acids in length, along with the restricting HLA-class I elements have been identified (Boon and van der Bruggen, 1996). Multiple attempts also have been made to identify breast cancer associated antigens although with limited success, using autologous breast cancer cells and tumor infiltrating/associated lymphocytes (TIL/TAL) (Peoples *et al.*, 1995; Linehan *et al.*, 1995). With the exception of melanoma, the use of an autologous system to study tumor-associated antigenic peptides is

usually complicated due to technical problems in maintaining long term growth of cytolytic TIL/TAL and lack of autologous PBL needed as feeder cells.

In this study, we successfully generated alloreactive cytotoxic T cells reactive against a HLA-class I matched breast cancer cell line. The cytolytic activity of these CTL was tissue specific and HLA-class I restricted. Furthermore, by growing the breast cancer cells in SCID mice, we were able to generate the large amount of breast cancer tissue needed for HLA-class I peptide isolation. More importantly, a biologically active, HPLC-separated HLA-class I peptide fraction was identified by testing the acid-eluted HLA-class I peptides for their ability to reconstitute the tumor antigenic epitope recognized by the CTL.

MATERIAL AND METHODS

Generation of T lymphocyte cell line and B-LCL

Peripheral blood mononuclear cells were obtained from a normal donor (BL.PBL) whose class I phenotype was HLA-A1,A3; B7,8 using density gradient centrifugation over Ficoll-Paque (Pharmacia, Piscataway, NJ). The breast cancer cell line HBL-100 (HLA-A1,A2; B8,60) was obtained from the ATCC (Rockville, MD) and maintained in RPMI-1640 (Sigma, St. Louis, MO) based medium supplemented with Hepes, Na pyruvate, glutamine, penicillin, streptomycin (GIBCO BRL, Grand Island, NY) and 15% (vol/vol) fetal bovine serum (Biocell, Rancho Dominguez, CA). The T cell line BL.HBL-100 was generated by culturing 10⁶ BL.PBL with 1¹⁰ HBL-100 cells irradiated with 5,000 rad in 10ml RPMI-1640 based culture medium (CM) supplemented with 15% (vol/vol) of pooled human serum (C-Six, Mequon, WI) and 30IU IL2/ml (Chiron, Emeryville, CA). The medium was changed every 2–3 days. This T cell line was restimulated every 10–14 days as followed: 5¹⁰ CTL were cultured with 1¹⁰ irradiated HBL-100 cells and 10¹⁰ autologous feeder cells (BL.PBL irradiated with 3,000 rad).

The B-lymphoblastoid cell lines were either developed by EBV transformation of PBL (Hadley and Mohanakumar, 1992) or obtained from the cell repository of the American Society for Histocompatibility and Immunogenetics.

⁵¹Chromium release assays and Ab blocking

Standard 4 hr ⁵¹Cr-release assays were performed. Briefly, targets were labeled with 250 μ Ci of ⁵¹Cr (ICN, Costa Mesa, CA) in 0.3 ml of CM for 1 hr, washed 3 times with CM and then plated at 5¹⁰ cells/well in a U-bottom 96-well plate (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). Effector cells were added to the wells at the indicated effector:target (E:T) ratios to a final

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volume of 200 μ l/well and incubated at 37°C. CM was used in place of effectors for spontaneous lysis and 1% Triton (Mallinckrodt, St. Louis, MO) solution was used for maximal lysis. After 4 hr of incubation, the 96-well plate was centrifuged at 800g for 5 min; 50 μ l of the supernatant was harvested from each well and transferred to a 96-well counting plate containing 100 μ l of counting fluid (Wallac, Gaithersburg, MD) per well. The radioactivity were measured in a Wallac Microbeta Plus counter (Gaithersburg, MD). Results are expressed as % specific lysis which is (experimental lysis - spontaneous lysis)/(maximum lysis - spontaneous lysis). Error bars reflect the standard deviation from the data point. Antibody (Ab) blocking studies were performed by adding the Ab at the indicated concentration to the 51 Cr-labeled targets with subsequent incubation at 4°C for 30 min prior to incubation with effectors at 37°C. Acid elution studies were performed by treating the 51 Cr-labeled targets with a pH 3.0 solution for 2 min, followed by immediate neutralization with a basic solution then washing prior to incubation with effectors at 37°C. Cell viability after acid treatment was assessed by Trypan blue exclusion (Sigma) exclusion.

Phenotyping of HBL-100, PBL, other cell lines and CTL

HLA phenotyping of HBL-100 cells, PBL and other cell lines used in this experiment were done either by DNA analysis or standard serological microcytotoxicity assays at the HLA Laboratory of Barnes Hospital. Select cell lines were analyzed by FACS for surface expressions of HLA Class I, HLA Class II and HLA-A1 using MAb W6/32 (ATCC), KUIA2 (7) and anti-HLA-A1 (One Lambda, Inc., Canoga Park, CA) respectively, as the primary Ab and goat anti-mouse FITC as the secondary Ab. Cytotoxic T lymphocytes were tested for their surface expression of CD3, CD4, CD8 and TCR $\alpha\beta$ by staining with fluoresceinated or phycoerythrin conjugated Abs followed by flow cytometric analysis using a FACScan with LYSYS software (Becton Dickinson, Mountain View, CA).

Cancer growth and verification

The HBL-100 cells were grown in severe combined immunodeficient (SCID) mice (the Jackson Laboratory, Bar Harbor, ME) by injecting $2-5 \times 10^6$ cells in 0.5 ml of Hank's buffer solution (GIBCO BRL) s.c. into the flanks of the mice. Tumors were allowed to grow to 4-5 cm in diameter then excised and 10g were used for peptide isolation. A section of the tumor was fixed in 10% formalin (Sigma) and paraffin embedded. Paraffin-embedded tissues were sectioned at 5 μ m, attached to glass slides, and deparaffinized as described by Saarialho-Kere *et al.* (1993). Endogenous peroxidase activity was blocked by incubating the slides in 0.3% H_2O_2 for 30 min at room temperature. Mouse-anti-human vimentin MAb (BioGenex, San Ramon, CA) was used at 1:10,000 dilution. The cytokeratin antibody was a cocktail of mouse-anti-human cytokeratin MAb including MAK6 (Zymed, South San Francisco, CA) at 1:20 dilution, cytokeratin AE1/AE3 (Boehringer Mannheim, Indianapolis, IN) at 1:150 dilution, and cytokeratin CAM 5.2 (Becton Dickinson) at 1:150 dilution. All immuno-histochemical stainings were done following microwave antigen retrieval in 0.1 M citrate buffer. The primary Abs were incubated overnight at 4°C. The secondary antibody, a biotinylated horse-anti-mouse IgG (Vector), was used at 1:200 dilution. Immune complexes were detected using a Vectastain ABC Elite kit (Vector) with diaminobenzidine tetra hydrochloride (DAB) as the chromogenic substrate. Slides were counter stained with Harris hematoxylin (Sigma) and examined with a Nikon microscope (Garden City, NY). McCoy's medium (Sigma) was used as the control. Staining of the SCID mouse intestinal epithelial cells were done for comparison. Hematoxylin and eosin staining was also done.

Peptide purification

HLA class I bound peptides were isolated following the procedure described by Falk *et al.* (1991). Briefly, HLA class I molecules were affinity purified from detergent extracts of 10 g of HBL-100

tumor tissues harvested from SCID mice, using 2 MAbs against HLA class I framework determinant, W6/32 and PA2.6 (ATCC). Peptides were eluted from HLA class I molecules with 0.1% trifluoroacetic acid treatment. Acid-soluble material (m.w. < 10 kDa) was saved for protein concentration determination by quantitative amino acid composition analysis. Peptides were separated by reversed phase high performance liquid chromatography (RP-HPLC) using a gradient of increasing acetonitrile concentration (0 to 60% linear gradient in 60 min) over a C18 column (25 \times 0.46 cm, 5 μ m; Vydac, Hesperia, CA) and Spectra Physics (San Jose, CA). All separations were conducted at flow rate of 1 ml/min and 1 ml fractions were collected. Chromatographic separation was monitored at a UV wavelength of 218 nm with a variable wavelength detector (Spectra Chrom 100).

Reconstitution of tumor epitope by addition of peptide fractions to a HLA-A1 $^+$ B-lymphoblastoid cell line (B-LCL)

Thirty percent (v/v) of individual peptide fractions were pooled in groups of 5. These pooled fractions were reduced to a final volume of approximately 20 μ l by use of a Speed Vac Concentrator (Savant, Farmingdale, NY), resuspended in CM to a final volume of 150 μ l and the pH was adjusted to 7.0. A homozygous HLA-A1 $^+$ B-LCL was labeled with 250 μ Ci of 51 Cr for 1 hr, washed and then seeded at 5×10^3 cells/well in 50 μ l of CM in a 96 well U-bottom plate (Falcon, Becton Dickinson Labware); 50 μ l of pH 7.0 adjusted peptide fractions were added to the targets in triplicates and the mixtures were incubated at room temperature for 2 hr. Effectors were added to the wells at the indicated E:T ratio in a final volume of 200 μ l, and then standard 51 Cr-release assays were performed.

RESULTS

Phenotyping of HBL-100 and other cell lines used in this study

Listed in Table I are HLA phenotypes, determined by DNA sequence analysis or standard serological microtoxicity assays, of cell lines used. Since cancer cells can down regulate HLA-Class I surface expression (Maeurer *et al.*, 1996), the cell lines which had HLA phenotyping assessed by DNA analysis were stained with HLA-class I MAb W6/32, HLA-class II antibody KUIA2 and

TABLE I - HLA PHENOTYPING OF CELL LINES USED

Cell line	A	B	DR
HBL-100 ¹	1, 2	8, 60	15, 17
MM.BCL	1, 33	7, 35	ND ²
CW.BCL	1, 33	49, 57	ND
SK-BR-3	11	40, 18	ND
ZR-75-1	11	35, —	ND
BL.PBL/BL.LCL ¹	1, 3	7, 8	2, 3
EFW145.LCL	1, 68	53, 58	12, 15
E4181324.LCL ¹	1	52	15
MM.LCL	1, 33	7, 35	ND
PC3	1, 9	—	ND
CACO2	1, 2	8, —	ND
HJX.KCL	1, 3	8, 13	7, 17
HTB-33	1, 11	5, 40	ND
HTB-39	1, 3	7, 13	ND
HTB-63	1, 24	13, 15	ND

¹Cell lines obtained from the ATCC: HBL-100, SK-BR-3 and ZR-75-1, breast cancer; PC3, prostate cancer; Caco-2 and HTB-39, colon adenocarcinoma; HTB-33, cervical carcinoma; HTB-63, melanoma. MM.BCL and CW.BCL are breast cancer cell lines established in our laboratory from primary tumor. BL.LCL, EFW145.LCL, E4181324.LCL and MM.LCL were B-LCLs established in our laboratory. E4181324.LCL was obtained from the American Society for Histocompatibility and Immunogenetics. Unless otherwise denoted by ¹ (¹ was performed by DNA sequencing), HLA phenotype was determined by standard serological microtoxicity assays. —ND, not done.

HLA-A1 Ab and analyzed by FACS to confirm their surface expression of HLA molecules. Representative FACS analysis is shown in Figure 1, confirming HLA-class I surface expression of the cell lines.

CD8⁺ lymphocytes are the predominant cell type

The polyclonal T lymphocyte bulk line BL.HBL-100 was generated as described in Material and Methods. To characterize these lymphocytes, FACS analysis was performed to assess their surface molecules. After 4 stimulations, the phenotypes of the T cells in this bulk line were as follows: 1. 59% CD8⁺ and 38.27% CD4⁺, and 2. greater than 99% CD3⁺ and TCR α/β ⁺. After 12 stimulations, the T cell line was predominantly CD8⁺ (98.54% CD8⁺ vs. 0.18% CD4⁺).

BL.HBL-100 CTL lysis of HBL-100 is CD8-mediated and HLA-Class I restricted

BL.HBL-100 CTL were tested for their ability to lyse the original stimulator cells, HBL-100, and also K562, a human natural

killer cell target. BL.HBL-100 CTL were able to lyse HBL-100 cells but not K562 (45% lysis vs. 2% lysis at an E:T ratio of 10:1), (Fig. 2). Anti-CD8 Ab significantly inhibited lysis of the target by 70%. Furthermore, W6/32 an anti-HLA-class I framework antibody, but not KuIA2 an anti-HLA-class II antibody, significantly blocked the lytic activity of BL.HBL-100 CTL against HBL-100 cells by 60% (Fig. 3).

The cytotoxic activity of BL.HBL-100 CTL is HLA-A1 restricted

To assess whether the lytic activity of the BL.HBL-100 CTL is HLA-A1 restricted, the T cells were used in a ⁵¹Cr-release assay against a panel of breast cancer cells. As shown in Figure 4a, only

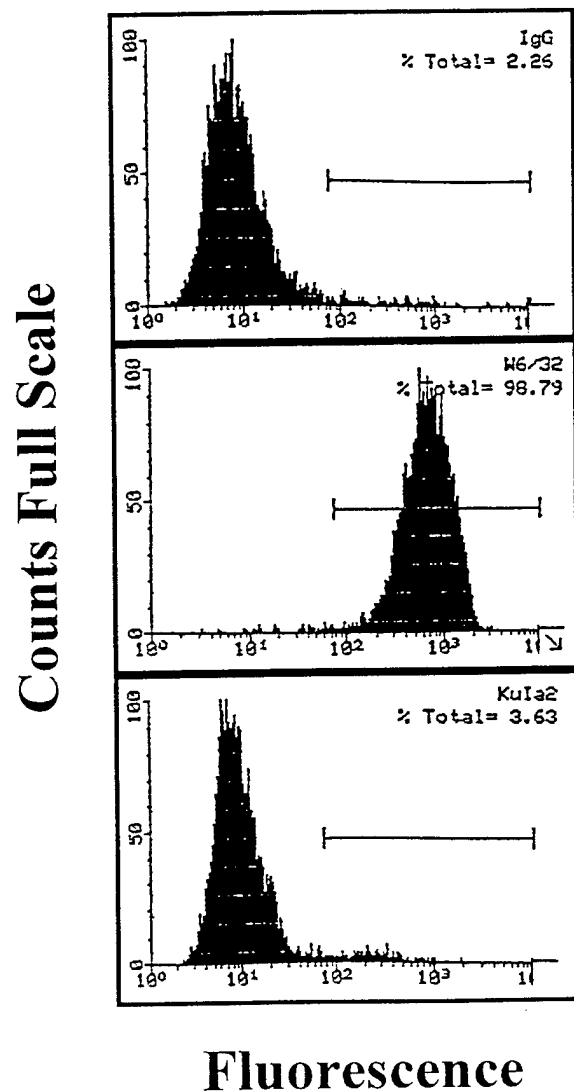


FIGURE 1 – Surface expression of HLA-class I molecules on HBL-100 cells. FACS analysis of HBL-100 cells was performed after the cells were incubated with (a) IgG, (b) W6/32, and (c) KuIA2 as the primary Abs (mouse-anti-human) and goat-anti-mouse FITC as the secondary Ab.

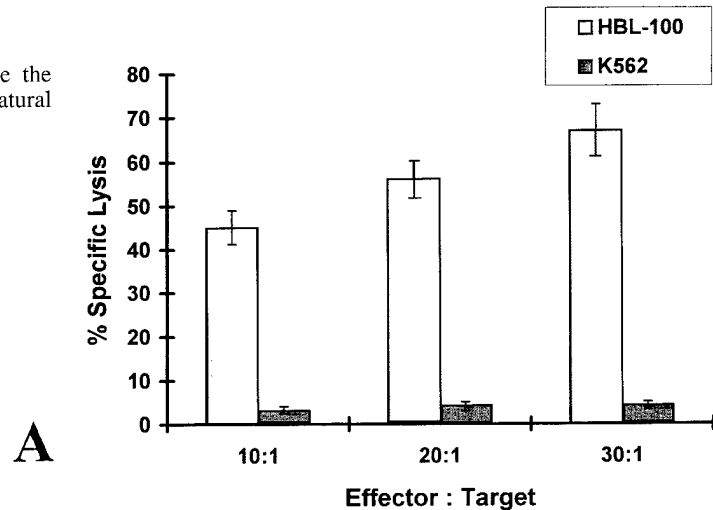


FIGURE 2 – Lysis of HBL-100 by BL.HBL-100 CTL. HBL-100, but not K562, cells are recognized and lysed efficiently by BL.HBL-100 CTL. The lytic activity is titratable.

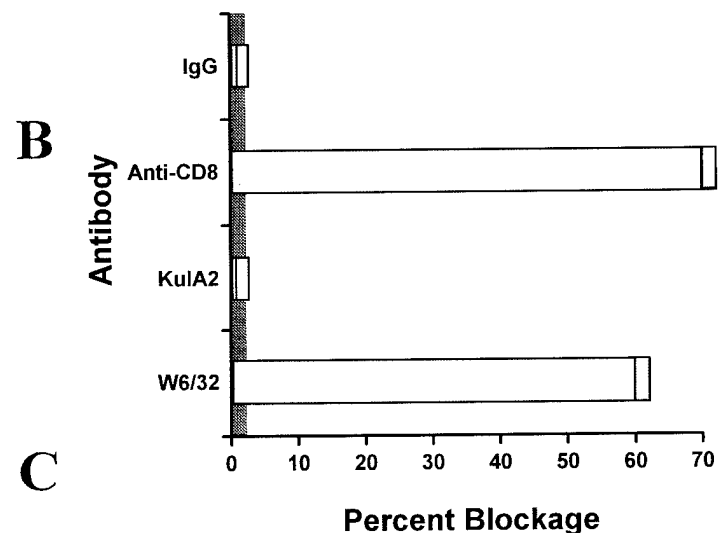


FIGURE 3 – Inhibition of target lysis by anti-CD8 and anti-HLA-class I MAbs. ⁵¹Cr release assay was performed at an E:T ratio of 10:1. Anti-CD8 and anti-HLA-class I MAbs but not IgG or KuIA2 an anti-HLA-class II Ab, blocked the lytic activity of BL.HBL-100 against HBL-100 cells by 69% and 60%, respectively. All Abs were used at a concentration of 25 μ g/ml. Lysis of HBL-100 by BL.HBL-100 in this experiment at an E:T ratio of 10:1 was 40% in the absence of Abs. Results are representative of duplicate experiments.

breast cancer cells which expressed HLA-A1 (MMBCL and CWBCL) was recognized and lysed by these CTL (24–39% specific lysis at an E:T ratio of 10:1) and non-A1 breast cancer cells (SKBR3, ZR75-1) were not lysed. Moreover, the lytic activity of the CTL against HLA-A1⁺ breast cancer cells was significantly blocked (65%) by an anti HLA-A1 MAB, indicating that the restriction element was HLA-A1 (Fig. 4b).

BL.HBL-100 cytotoxic activity is tissue specific

To determine the tissue specificity of the BL.HBL-100 CTL, the CTL were tested for their ability to lyse a panel of HLA-A1⁺ normal and malignant cell lines. The lytic activity of these CTL was found to be breast cancer specific. At an E:T ratio of 15:1, HBL-100 was lysed at 40% while other cell lines including HLA-A1⁺ normal kidney epithelial cells (HJX.KCL), prostate cancer (PC3), colon

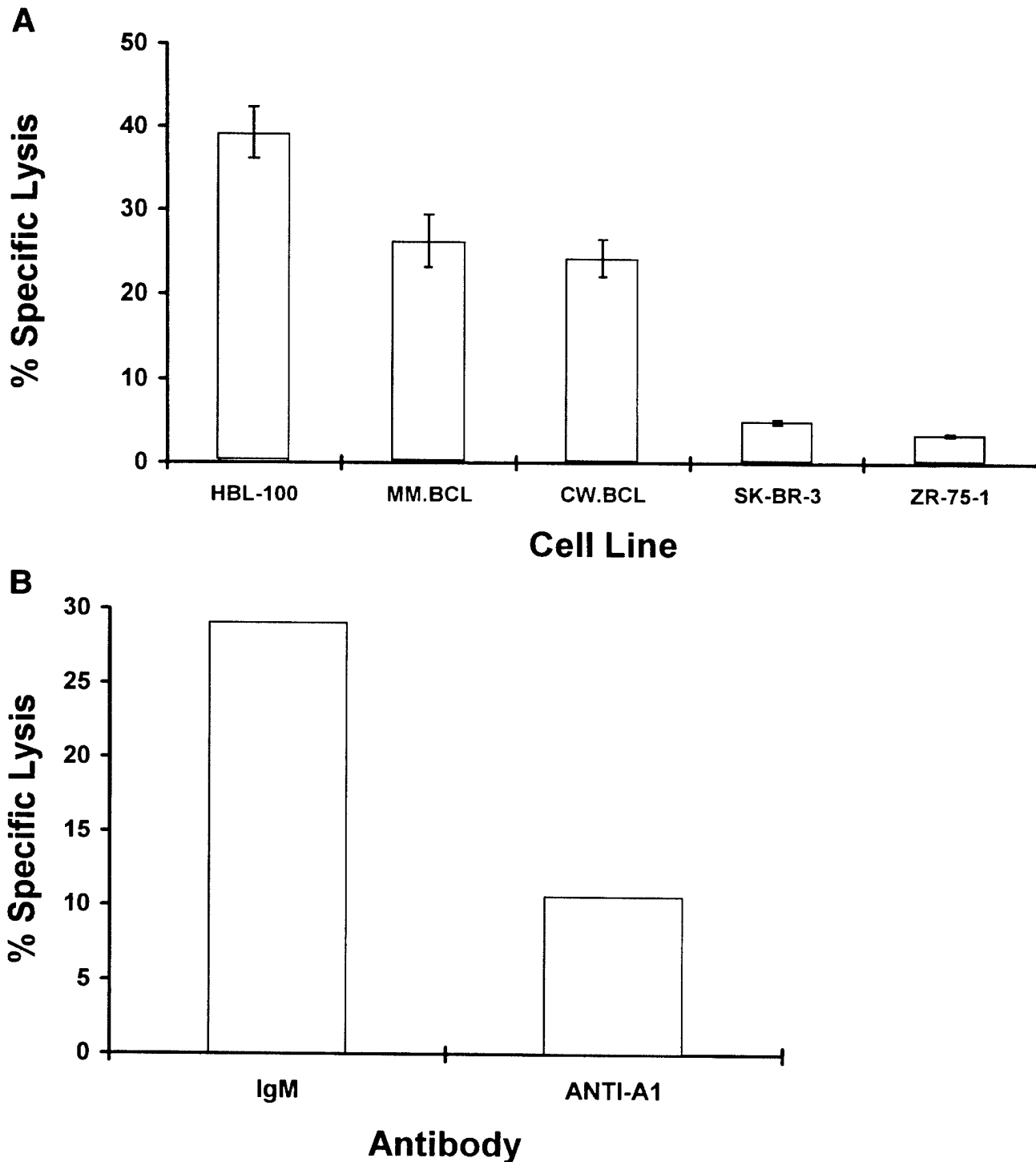


FIGURE 4 – BL.HBL-100 lytic activity is HLA-A1 restricted. ⁵¹Cr release assay was performed at an E:T ratio of 10:1. (a) Only breast cancer cells which carried the HLA-A1 phenotype were recognized by the CTL. (b) An anti HLA-A1 Ab but not IgM, significantly blocked lysis of MM.BCL cells by the CTL. All Abs were used at a concentration of 25μg/ml. Lysis of MM.BCL by BL.HBL-100 in this experiment at an E:T ratio of 10:1 was 28% in the absence of antibodies. Results are representative of duplicate experiments.

adenocarcinoma (CaCo2), cervical carcinoma (HTB33), B lymphoblastoid cells (BL.LCL, EFW145.LCL, E4181324.LCL and MM.LCL); Daudi; and K562 were not significantly lysed (Fig. 5).

Peptides are involved in CTL recognition of the HBL-100 cells

It is generally accepted that CTL recognize endogenous antigenic peptides presented on the cell surface by HLA-class I molecules (Jorgensen *et al.*, 1992) and hence, acid stripping of the antigenic peptides from the HLA-class I-peptide complexes should abolish CTL recognition of their target. Therefore, to confirm that HLA-class I peptides were involved in the CTL recognition of the HBL-100 cells, the HBL-100 cells were subjected to acid elution of HLA bound peptides. Cell viability after acid treatment followed by immediate neutralization with a basic solution was greater than 95% as assessed by Trypan blue dye exclusion. Without cell surface peptides, there was no recognition of the HBL-100 cells by the tumor specific CTL (Fig. 6).

HBL-100 tissues harvested from SCID mice are of human origin

To ascertain that the cancer tissues which were harvested from SCID mice are of human origin, immuno-histochemical staining of the tissues with mouse-anti-human vimentin and keratin was performed and compared to staining of SCID mouse intestinal sections (negative control). Cell morphology was examined in hematoxylin and eosin stains of the tumor sections which showed the cells to have round to oval nuclei, vesicular chromatin, eosinophilic centrally placed nucleoli, moderate pleomorphism, moderately abundant cytoplasm and frequent mitotic figures consistent with poorly differentiated carcinoma. Immuno-histochemical staining using mouse-anti-human vimentin and keratin Abs confirmed the human origin of the tumor (Fig. 7).

Reconstitution of the tumor antigenic epitope recognized by BL.HBL-100 CTL

To assess if the HLA-A1 restricted epitope(s) recognized by BL.HBL-100 CTL could be isolated from peptides bound to

HLA-class I molecules expressed on the cell surface of HBL-100 cells, HBL-100 was grown in SCID mice and 10 g of tumor were harvested and used for peptide isolation. HLA-class I molecules were affinity purified from the HBL-100 tissue. Peptides were eluted from HLA-class I molecules and separated by RP-HPLC (Fig. 8). Pooled peptide fractions were incubated with an HLA A1⁺ B-LCL (E4181324 B-LCL), a A1⁺ homozygous B-LCL normally not recognized by the CTL (Fig. 5) and tested for their ability to reconstitute the tumor antigenic epitope(s) as evidenced by lysis of the peptide-pulsed B-LCL. The biologically active peptides were found in a pool of fractions 36 to 40, as evidenced by 16% specific lysis of the peptide-pulsed B-LCL at an E:T ratio of 20:1 (Fig. 9a). Assays using these individual fractions showed antigenic peptides located in fraction 40, as evidenced by 47% lysis of the peptide-pulsed B-LCL at an E:T ratio of 40:1 (Fig. 9b). Preliminary studies using mass spectrometry revealed that fraction 40 contained several small peptides with molecular mass range 800–1,100 thus indicating the presence of peptides of 8–10 aa in length (not shown).

DISCUSSION

Tumor immunology has been a subject of great interest for over a century, but progress has been limited until recently, when improved molecular understanding of human tumor immunity and some technical advances made informative experiments more feasible. Anecdotal reports of spontaneous regression of melanoma (Whicker *et al.*, 1980, Baldo *et al.*, 1992) suggested a possible immune response to tumor. The immune response to certain cancers was further confirmed when tumor specific, HLA-class I restricted CTL were isolated from the tumor tissues, tumor-draining lymph nodes, and malignant effusions (Linehan *et al.*, 1995; Chen and Hersey, 1992). However, the immune response to tumor is apparently inadequate since the majority of patients whose tumors are not resected at an early stage will go on to develop

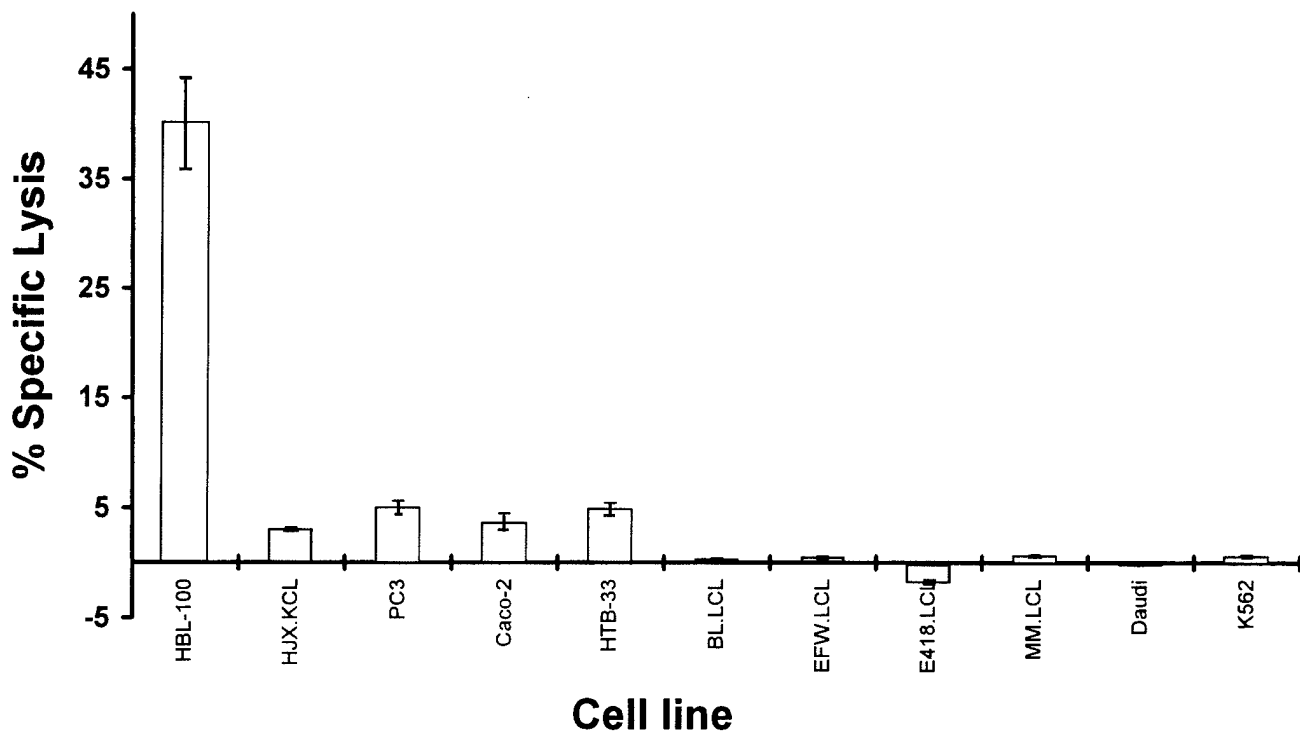


FIGURE 5 – BL.HBL-100 CTL lytic activity is tissue specific. ⁵¹Cr-release assay was done at an E:T ratio of 10:1. All cell lines used in this assay expressed the HLA-A1 phenotype. However, only breast cancer cells were significantly lysed by the CTL. HJX.KCL: normal kidney epithelial cell line; PC3: prostate carcinoma; Caco-2: colon carcinoma; HTB-33: cervical carcinoma; BL.LCL, EFW145.LCL, E4181324.LCL, MM.LCL: B-lymphoblastoid cell lines; Daudi: a lymphokine activated cell target; and K562: a natural killer cell target.

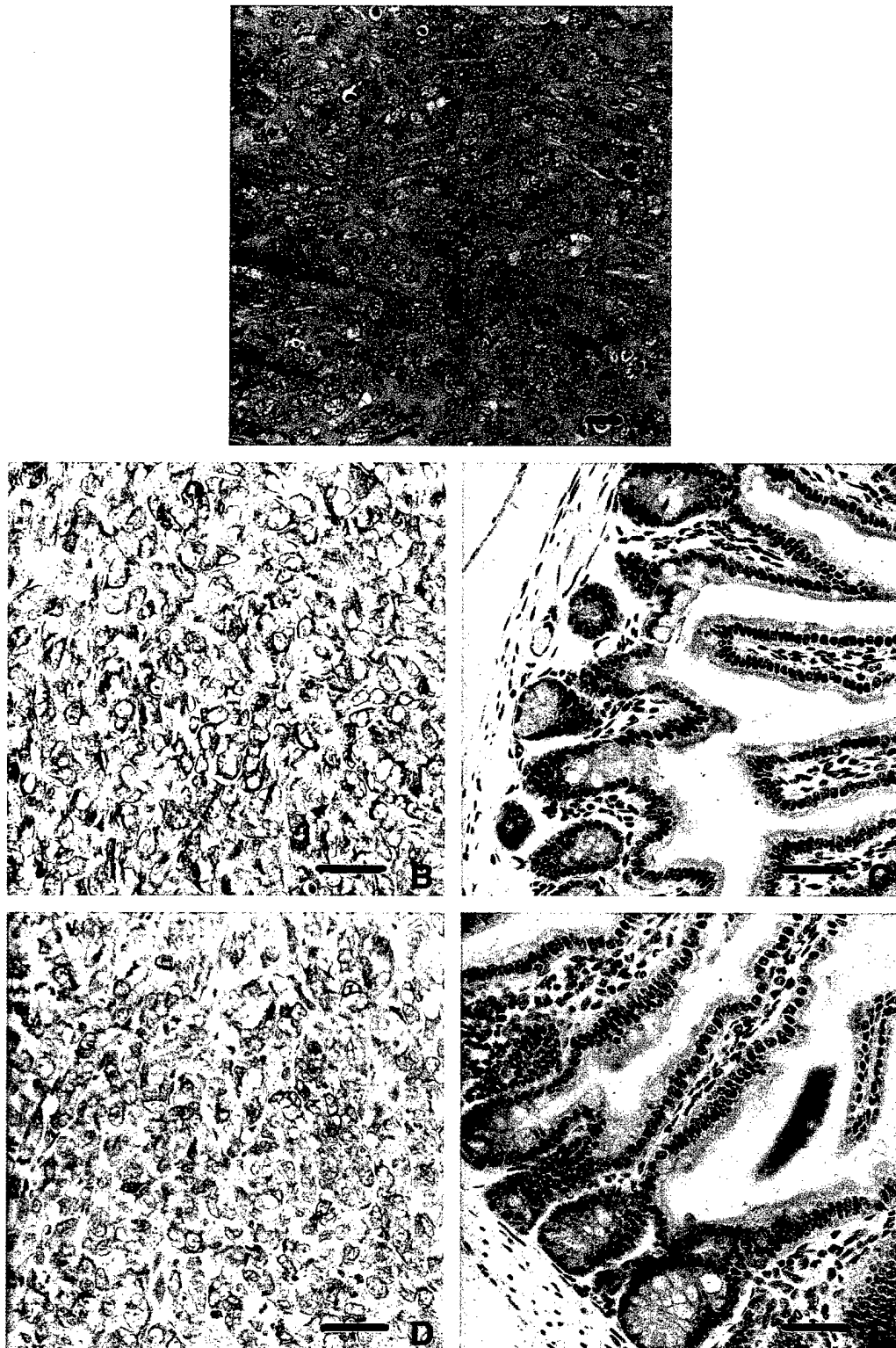


FIGURE 7 – H&E and immuno-histochemical stainings of HBL-100 tissues harvested from SCID mice. (a) H&E stainings showed the HBL-100 cells to have moderate pleomorphism and frequent mitotic figures consistent with poorly differentiated carcinoma. (b) Anti human-vimentin staining showed strong, diffuse cytoplasmic positivity in HBL-100 cells but not (c) SCID mouse intestine. (d) Anti human-keratin staining was again strongly positive in HBL-100 cells but not (e) SCID mouse intestinal epithelium. Scale bar, 62.5 μ m.

(Peoples *et al.*, 1995; Linehan *et al.*, 1995) and melanoma (Cox *et al.*, 1994).

The lytic activity of BL.HBL-100 CTL against HLA-A1⁺ breast cancer cell lines was significantly blocked by anti-CD8, anti-HLA-

class I framework, and anti-HLA-A1 MAbs (Figs. 3, 4). These results suggested that the lysis of the breast cancer cells in our study is mediated by cytotoxic T lymphocytes and is HLA-A1 restricted.

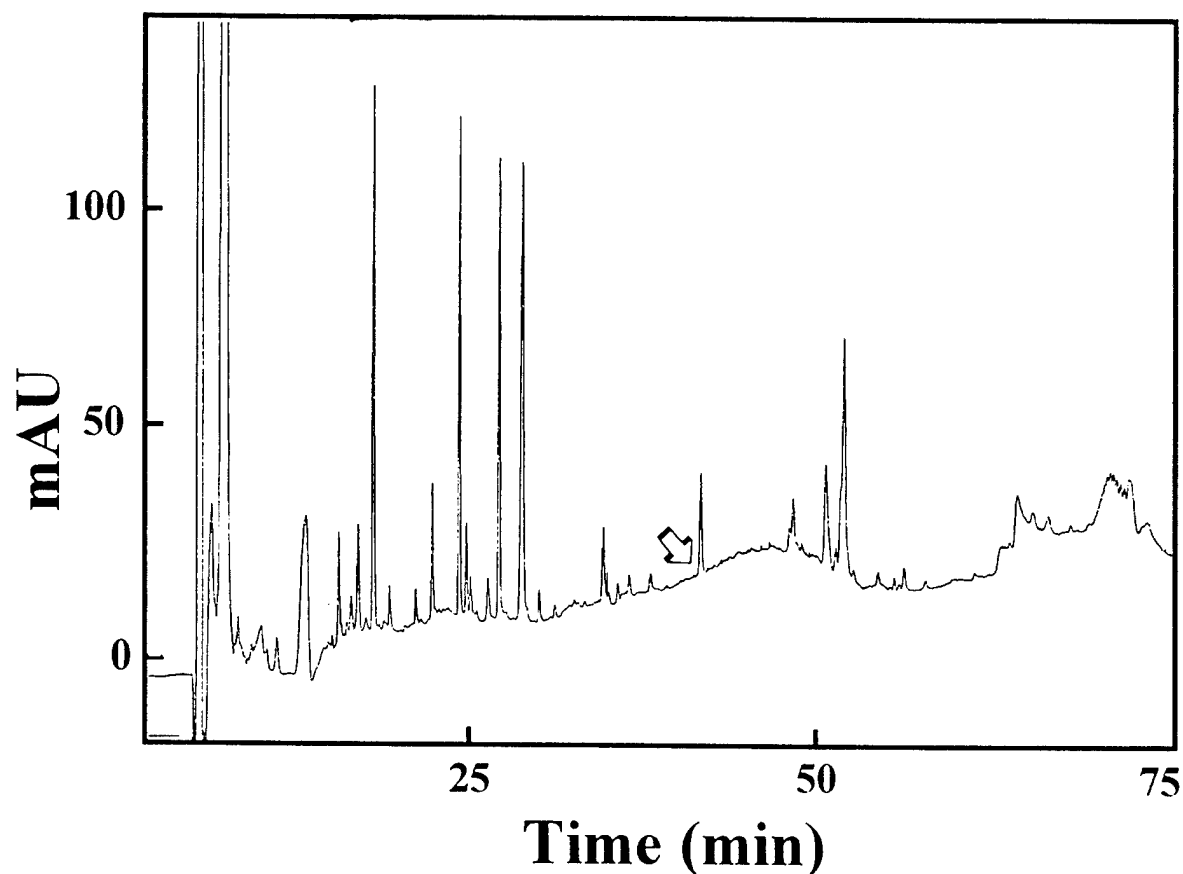


FIGURE 8 – RP-HPLC profile of HBL-100 peptides eluted from HLA-class I molecules. The biologically active fraction 40 is marked by the arrow.

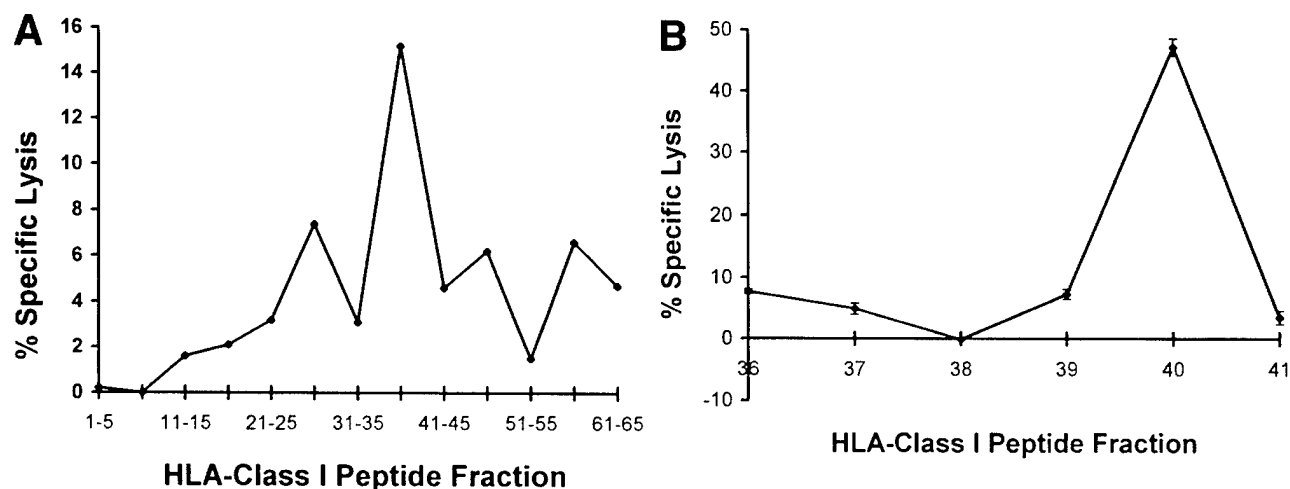


FIGURE 9 – Reconstitution of the tumor antigenic epitope(s) recognized by BL.HBL-100 CTL. Pooled HLA-Class I peptide fractions were tested for their ability to reconstitute the tumor antigenic epitope as evidenced by lysis of the peptide-pulsed E4181324 B.LCL by the CTL. (a) ^{51}Cr release assay was done at an E:T ratio of 20:1 showing peak lysis occurred at pooled fractions 36–40. (b) Assays using these individual fractions at an E:T ratio of 40:1 showed the biologically active peptides residing in fraction 40.

As mentioned previously, CTL are known to recognize endogenous peptides presented on the cell surface by HLA-class I molecules (Jorgensen *et al.*, 1992). To demonstrate that peptides are involved in CTL recognition of the target cells in our study, HBL-100 cells were subjected to acid treatment to elute the peptides from HLA molecules. Without peptides on the cell surface, there was no recognition of the target cells by the CTL (Fig. 6). Furthermore, MM.BCL and CW.BCL, the 2 HLAA1⁺

breast cancer cell lines were also subjected to low pH treatment that eluted class I bound peptides prior to ^{51}Cr -release assay. Depletion of peptides abrogated lysis of these 2 cell lines by BL.HBL-100 (data not shown). This data suggested that peptides bound to HLA class I are involved in the recognition by BL.HBL-100.

Acid elution of HLA-class I peptides was utilized successfully by several investigators to identify tumor-associated antigenic

peptides (Peoples *et al.*, 1995; Cox *et al.*, 1994). This method was also utilized in our laboratory to identify a kidney-specific peptide (Poindexter *et al.*, 1995). However, massive number of cells are usually needed for acid elution of HLA-class I peptides. The requirement for a large number of cells could be a challenging problem since operating room specimens are usually completely utilized by the pathologist for staging and histological/immunological classification of the tumors; moreover, establishment and propagation of breast cancer cells *in vitro* from primary tumor is arduous. We circumvented this problem by growing breast cancer cells in SCID mice. This method allowed us to obtain large supply of cancer tissues necessary for our study. Utilizing HLA-class I HPLC separated peptide fractions obtained from these tissues, we were able to identify the biologically active peptide fraction which reconstituted antigenic epitopes recognized by the breast cancer specific, HLA-class I restricted CTL.

We utilized CTL from a normal donor directed against an HLA class I matched malignant cell line to identify tumor-associated antigen(s). This method offers several advantages over the conventional method of using the patient's cells, in that the PBL and feeder cells are readily available, and a large tumor volume could be obtained for peptide isolation by growing the tumor in SCID mice. One potential draw back of our method is that it does not distinguish tumor-associated from tumor-specific antigenic peptides. All breast tissue peptides presented on the cell surface with the appropriate HLA-class I restricting element could potentially be recognized by the CTL. However, with the exceptions of mutated ras antigenic peptides in colon and pancreatic cancer and mucin-derived antigenic peptides in breast, ovarian, colon, and pancreatic cancer (Kotera *et al.*, 1994; Peace *et al.*, 1994), antigenic peptides identified thus far are tumor-associated, not specific, antigens in that they are present in normal tissues as well.

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Human dendritic cell mediated cytotoxicity against breast carcinoma cells in vitro

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Abstract: Dendritic cells (DC) are an important subset of antigen-presenting cells characterized by their potent capacity to activate immunologically naïve T cells. However, their role in effector function in tumor resistance is less well characterized. We report here that activated human peripheral blood DC acquire a potent antitumor effect against breast cancer cell lines in vitro, leading to growth inhibition and apoptosis of the tumor cell. The antitumor effect of DC was augmented by proinflammatory stimuli induced by lipopolysaccharide (LPS) treatment. Tumor necrosis factor α (TNF- α) produced after DC activation was responsible for the antitumor activity of DC. Interferon- γ , interleukin-15, or LPS treatment of DC markedly augmented the effector function of DC against most of the breast cells, indicating heterogeneity of the tumor and its susceptibility to cytokine-mediated damage. Treatment of LPS-activated DC or cell-free supernatant with anti-human TNF- α significantly reduces the antitumor effect against the tumor cells tested. These results suggest that in addition to their predominant role as immune regulatory cells, DC could serve as innate effector cells in tumor immunity. *J. Leukoc. Biol.* 72: 312-320; 2002.

Key Words: apoptosis · TNF- α · IFN- γ · LPS

INTRODUCTION

Dendritic cells (DC) are a distinct population of bone marrow-derived cells that play a key role in initiating immune responses by uptake, processing, and presenting antigens [1-3]. Recent reports have demonstrated that human DC can efficiently present antigens to CD8 $^{+}$ T cells derived from apoptotic cells in vitro [4, 5]. This may result in "cross priming," where antigens derived from dying tumor cells are presented by host antigen-presenting cells (APC) to CD8 $^{+}$ T cells. Because of their exceptional capacity to activate naïve T cells, DC loaded with antigens in the form of peptides, cell lysates, or RNA have also been used to induce cell-mediated, immune responses against tumors [6-9]. Preliminary results from clinical trials using DC pulsed with peptides derived from tumor antigens or vaccination with tumor cell-DC hybrids have shown promising results [10-13].

The immune surveillance role of DC has been documented in several systems, which involves the secretion of important immunoregulators such as tumor necrosis factor α (TNF- α) [14]. DC are found to infiltrate to the areas surrounding human solid tumors, and the density of their infiltration has been correlated with the condition of the disease [15-17]. Besides the classical antigen-presenting role of DC to T cells, the direct role of DC against tumor has been reported in recent times [18-21].

In this communication, we studied the direct interaction of human DC to a variety of breast cancer cell lines. DC showed variable levels of cytotoxicity as well as growth inhibition of breast cancer cell lines. Pretreatment of DC with interferon- γ (IFN- γ) and interleukin-15 (IL-15) or lipopolysaccharide (LPS) augmented DC-mediated growth inhibition and cytotoxicity. This antitumor effect of DC is associated with apoptosis of tumor cells and provides a new mechanism by which DC mediate antitumor function in breast cancer.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF), TNF- α , IL-15, and IFN- γ as well as all antibodies used in this study were purchased from Pharmingen BD Biosciences (San Diego, CA). LPS was purchased from Sigma Chemical Co. (St. Louis, MO).

Cells

The MCF-7 (breast adenocarcinoma), BT-20 (breast carcinoma), HBL-100 (breast and lung carcinoma), MDA-MB-231 (breast adenocarcinoma), MDA-MB-415 (breast adenocarcinoma), BT-474 (breast ductal carcinoma), MDA-MB-175 (breast ductal carcinoma), and MRC-5 (fibroblast) cell lines were purchased from American Type Culture Collection (Manassas, VA). B-lymphoblastoid cell lines (B-LCL) immortalized by Epstein-Barr virus were established in the laboratory. The cell culture medium used was RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies), and 10 μ g/ml ciprofloxacin.

Isolation of DC

Peripheral blood DC were purified from peripheral blood mononuclear cells (PBMC) by incubating the adherent cell population overnight at 37°C, 5%

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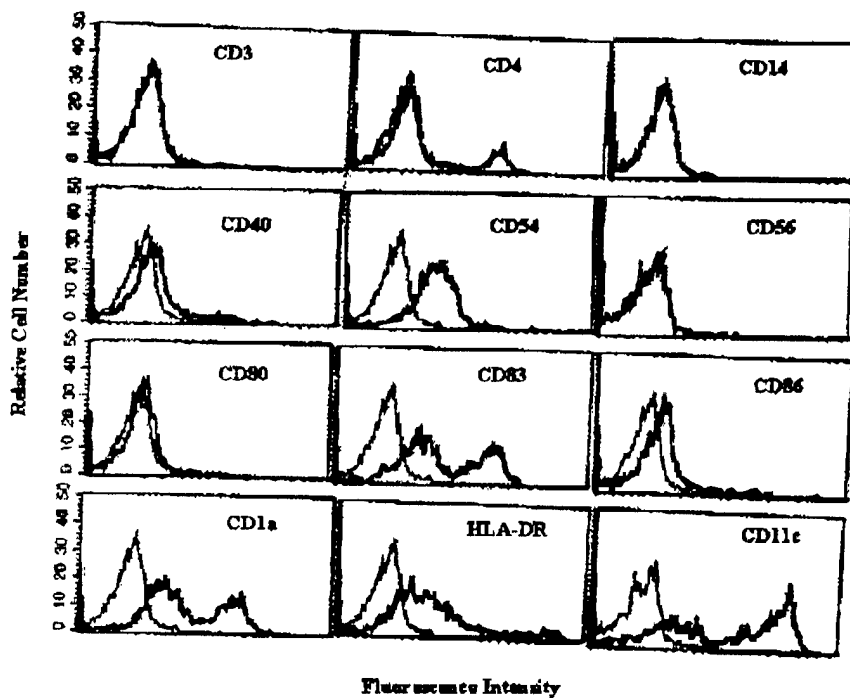


Fig. 1. The phenotype of human peripheral blood-derived DC. PBMC were adhered for 2–4 h at 37°C, 5% CO₂. The nonadherent cells were removed, and adherent cells were washed and incubated at 37°C, 5% CO₂, for another 18–24 h in complete medium. After overnight incubation, the DC were isolated from the loosely adherent cell population by depleting CD3+, CD14+, CD19+, and CD56+ cells using specific mAb and goat anti-mouse, polyvalent Ig-coated immunomagnetic beads. Expression of the indicated antigens was analyzed by single color flow cytometry using PE- or FITC-conjugated mAb. Data represent one individual donor. Similar results were obtained from other unrelated donors used in the study.

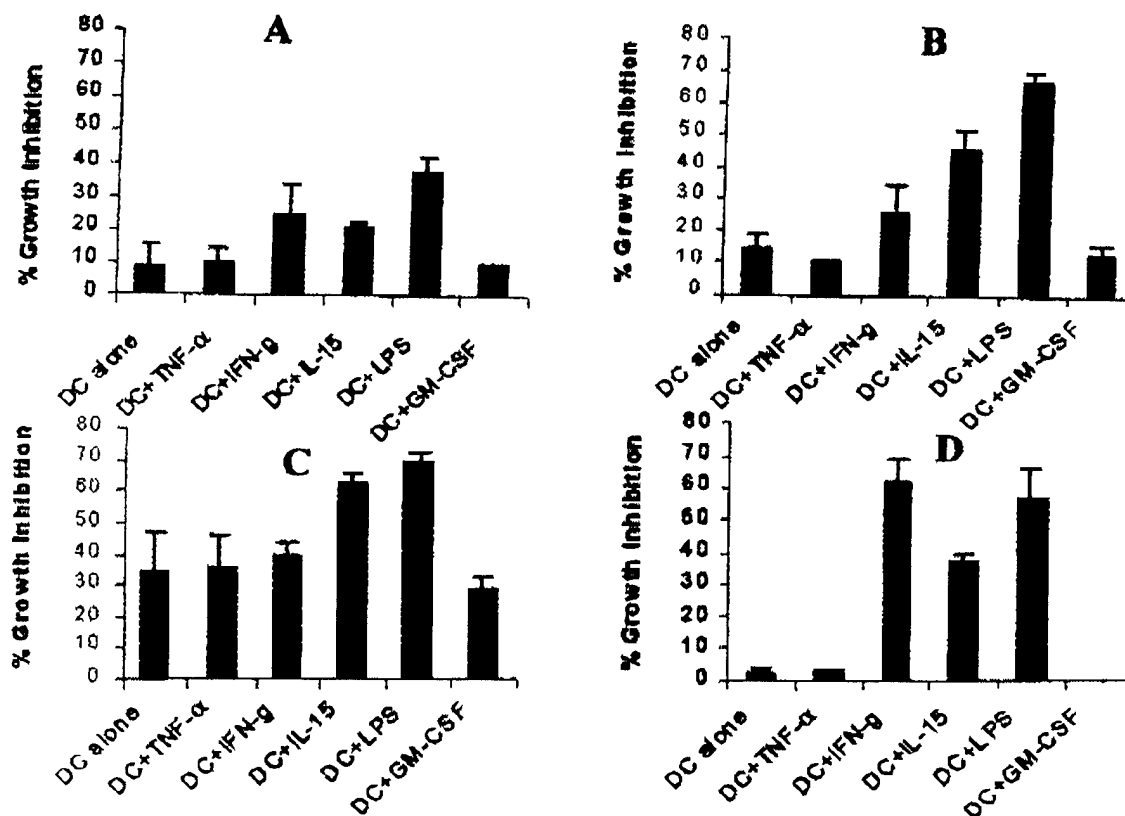


Fig. 2. DC-mediated growth inhibition of human breast tumor lines in vitro. DC (5×10^4 /well) were cultured in 96-well plates with or without indicated cytokines or LPS at 37°C, 5% CO₂. At 24 h, the cells were washed ($\times 3$) with complete medium and resuspended in the same medium. Indicated tumor lines MCF-7 (A), HBL-100 (B), MDA-MB-231 (C), MDA-MB-415 (D), LCL, or MRC-5 at 5×10^3 cells/well were added to the wells containing naive or activated DC and cocultured for 24 h at 37°C, 5% CO₂, and for an additional 24 h, with ³H₃ thymidine before being harvested. The results are presented as the mean percentage of inhibition of tumor cell proliferation \pm SD of triplicate wells. Thymidine incorporation into DC alone was less than 700 cpm. Representative of five experiments is shown here.

CO₂. The peripheral blood DC generally adhere along with the monocytes but become nonadherent after overnight incubation. The DC population was isolated from this heterogeneous, nonadherent population of cells by treatment with monoclonal antibodies (mAb) to CD3, CD14, CD19, and CD56 plus goat anti-mouse immunoglobulin (Ig)-conjugated magnetic beads (Dynal, Oslo, Norway). After four rounds of treatment, DC were purified from a harvested, nonadherent population. The purity of DC was determined by surface expression of DC-specific markers such as CD1a, CD11c, CD83, and human leukocyte antigen (HLA)-DR and was analyzed in a FACScan (Becton Dickinson, San Jose, CA). The purity of DC generated by this method was >98% and was free from monocytes and other contaminating leukocytes. Cell viability was >98% as determined by trypan blue dye exclusion.

The enriched DC show surface expression of CD11c, CD1a, CD83, and HLA-DR and do not express CD3, CD14, CD19, or CD56, indicating effector cells used for the functional analysis are blood DC. Figure 1 represents the surface phenotype of one DC preparation used in the study. Up-regulation of costimulatory molecules (CD40, CD80, CD86), CD83, and HLA-DR was observed in DC after incubation with indicated cytokines or LPS, and the expression of CD11c and CD1a remains unchanged (data not shown). The expression of CD83 in our DC preparation is similar to Fanger et al. [19], who also observed that fresh human peripheral blood-derived DC start expressing CD83 after overnight incubation with media.

Tumor growth inhibition assay

DC (5×10^4 cells per well) were cultured in 96-well plates with medium alone or in the presence of recombinant human TNF- α (500 pg/ml), IFN- γ (1000 U/ml), IL-15 (200 pg/ml), GM-CSF (1000 U/ml), or LPS (10 μ g/ml). After 24 h,

the cells were washed ($\times 3$), and tumor cells (5×10^3) were added to the wells. Plates were incubated at 37°C, 5% CO₂, for 24 h and then pulsed with 1 μ Ci/well of [³H]thymidine (ICN, Irvine, CA). The plates were harvested, and thymidine incorporation was assessed by means of liquid scintillation counter (Wallac, Gaithersburg, MD). The data are presented as the percentage of inhibition calculated from the following formula: % Inhibition = $(1 - \text{test cpm}/\text{control cpm}) \times 100$, where test cpm is thymidine incorporation by tumor cells cultured with DC after various stimulations, and control cpm is the corresponding value of tumor cell only cultured in the absence of DC. DC with or without various stimuli did not incorporate a significant amount of radioactivity (less than 700), and the tumor cells usually incorporated 7500–85,000 cpm, depending on the type of tumor line.

In some experiments, DC were plated in 96-well plates and incubated with or without LPS (10 μ g/ml), IFN- γ (1000 U/ml), or IL-15 (200 pg/ml) for 24 h. After 24 h, the supernatant was collected and cells were washed with complete medium ($\times 3$). The cells were treated with a saturating concentration of antibodies to human TNF- α , human IL-12 (p40/p70), or human IFN- γ for 1 h before addition of tumor cells at an effector:target (E:T) ratio of 10:1. The supernatant generated from DC after LPS activation was added to separate 96-well plates and treated with antibodies to human TNF- α or human IL-12 (p40/p70) for 1 h before addition of tumor cells. The incubation of tumor cells with DC alone or DC-derived supernatant was the same as before. The cells were pulsed with [³H]thymidine for the last 24 h of incubation before being harvested.

Intracellular staining of TNF- α in DC

Peripheral blood-derived DC were cultured for 24 h with media alone or media supplemented with TNF- α , GM-CSF, IFN- γ , IL-15, or LPS. The cells were

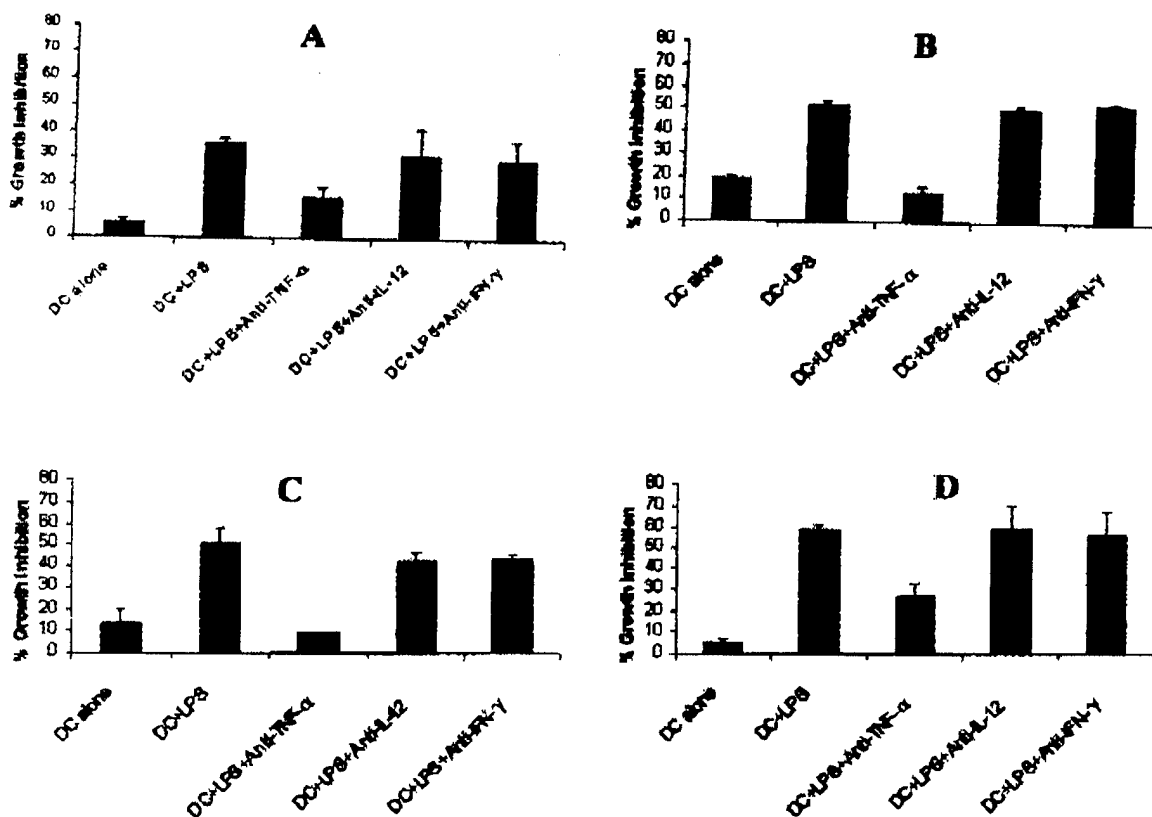


Fig. 3. Enhancement effect of antitumor potential of LPS-activated DC is mediated by soluble factor. DC (5×10^4 /well) were incubated with or without LPS (10 μ g/ml). At 24 h, the supernatants were collected, and DC were washed ($\times 3$) with complete medium and treated with anti-human TNF- α , anti-human IL-12, or anti-human IFN- γ at a neutralizing concentration for 1 h before the addition of respective tumor targets. The DC and tumor cells MCF-7 (A), HBL-100 (B), MDA-MB-231 (C), or MDA-MB-415 (D) were incubated for 24 h at 37°C, 5% CO₂, and for an additional 24 h, with [³H] thymidine before being harvested. The results are presented as the mean percentage of inhibition of tumor cell proliferation \pm SD of triplicate determination. Representative of four experiments is shown here.

washed and fixed with 1% paraformaldehyde for 5 min at room temperature followed by treatment with α -gluco pyranoside (7 mg/ml) for 5 min at room temperature. The cells were washed and stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human TNF- α for 45 min and analyzed in a FACScan.

DC cytotoxic activity assay

The lytic activity of peripheral blood DC was measured by 18-h ^{51}Cr -release assay. DC were cultured for 24 h in media alone or in the presence of GM-CSF (1000 U/ml), TNF- α (500 pg/ml), IFN- γ (1000 U/ml), IL-15 (200 pg/ml), or LPS (10 $\mu\text{g}/\text{ml}$) in 96-well plates at 37°C, 5% CO_2 . The cells were washed ($\times 3$) and resuspended in complete media before addition of radiolabeled tumor cells. Tumor cells, MRC-5, or B-LCL were labeled with 100 μCi ^{51}Cr for 1 h at 37°C, washed three times, and resuspended in complete media. Target cells (5×10^5) were added to the unstimulated and cytokine-stimulated DC at a fixed E:T ratio of 10:1 and incubated for 18 h at 37°C, 5% CO_2 . After incubation, the supernatant was collected, and ^{51}Cr release was measured in a γ counter. Percent-specific lysis was determined using the following formula: (experimental cpm - spontaneous cpm / maximum cpm - spontaneous cpm) $\times 100$.

Detection of apoptosis

We evaluated the ability of unactivated or activated DC to induce apoptotic cell death in breast cancer cell lines by binding FITC-conjugated Annexin V. Light-scatter characteristics were used to distinguish the tumor cells from the DC, such that only the tumor cells were counted in the analysis. After 8 h of incubation, the percentage of FITC-conjugated Annexin V-positive cells was analyzed by flow cytometry (Becton Dickinson). In some experiments, the incubation of tumor cells and various DC populations was extended for 24 h

followed by staining with Annexin V and propidium iodide (PI) and was analyzed by flow cytometry.

RESULTS

Growth inhibition effect of DC on breast tumor lines

To examine whether DC can affect the growth of breast tumor cells, we performed a 48-h growth inhibition assay by coculturing DC with a panel of breast tumor cell lines. Our data show that peripheral blood DC (unstimulated and cytokine- or LPS-stimulated) significantly inhibit the growth of MCF-7, HBL-100, MDA-MB-231, and MDA-MB-415 breast cancer cell lines (Fig. 2). No growth inhibition was observed in two unrelated B-LCL lines and also in fibroblast cell line MRC-5 (data not shown), indicating the growth inhibition effect of DC (activated and unactivated) on breast cancer cell lines is specific. Stimulation with IFN- γ , IL-15, or LPS significantly inhibited the growth of MCF-7 cells by 25%, 22%, and 36%, respectively. On the other hand, unstimulated DC could inhibit breast cancer cell growth by only 7%, which was not enhanced when tumor cells were treated with DC stimulated with TNF- α or GM-CSF. Pretreatment of DC with recombinant human

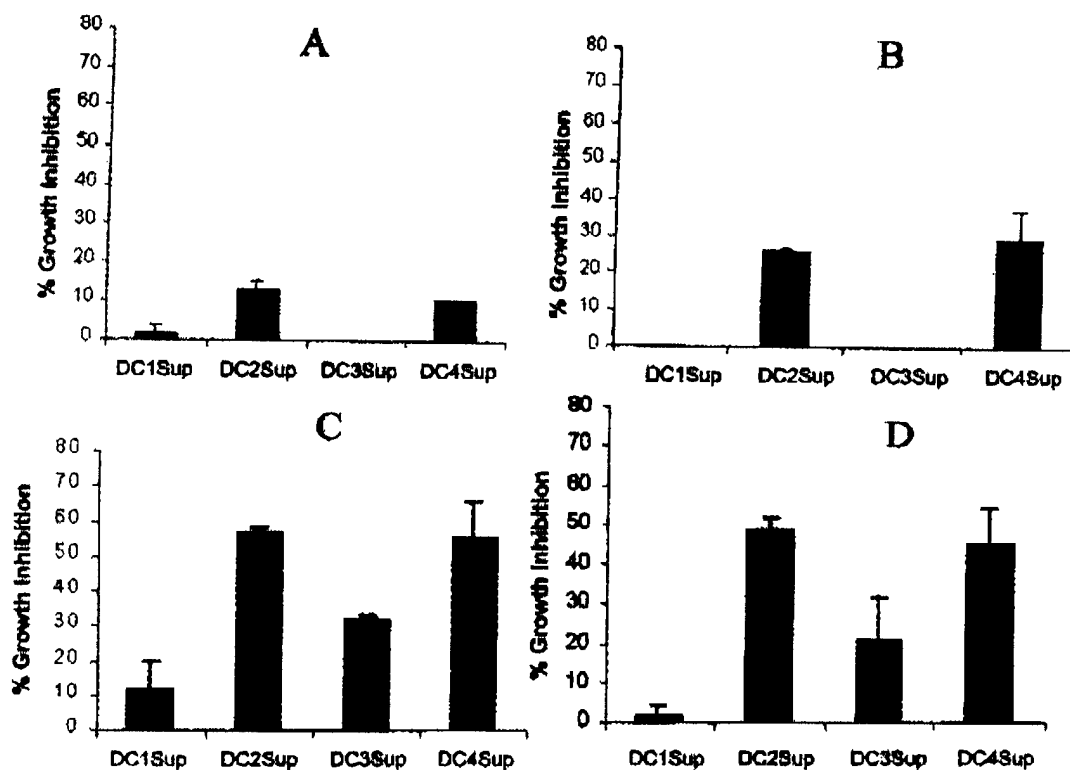


Fig. 4. Contact independent inhibition of tumor targets by soluble factors derived from LPS-activated DC. The cell-free culture supernatants generated from DC cultured with media alone (DC1Sup) or with LPS (DC2Sup) were filtered by a membrane with 0.45 μm pore size. The undiluted, LPS-activated, DC-derived supernatants were further treated with anti-human TNF- α (DC3Sup) or anti-human IL-12 (DC4Sup) antibody for 1 h in 96-well plates before the addition of respective tumor targets MCF-7 (A), HBL-100 (B), MDA-MB-231 (C), or MDA-MB-415 (D). The tumor cells were incubated with the supernatant at 37°C, 5% CO_2 , for 24 h and for an additional 24 h, with ^3H , thymidine before being harvested. The results are presented as the mean percentage inhibition \pm SD of triplicate determination. Representative of four experiments is shown here.

TNF- α or GM-CSF had no significant, stimulatory capacity on DC as compared with the effect of other cytokines and stimulatory agents (Fig. 2).

Role of soluble mediators on growth inhibition of breast cancer cells by activated DC

To determine the mechanism of the DC-mediated growth inhibition, DC were cultured with IFN- γ , IL-15, or LPS for 24 h. The coculture of activated DC and tumor cells was treated with a saturating concentration of anti-human TNF- α , anti-human IL-12, or anti-human IFN- γ antibody and was incubated for another 24 h prior to the addition of thymidine. Treatment of activated DC with anti-human TNF- α , but not with anti-human IL-12 or anti-human IFN- γ , abolishes the antitumor potential of cytokine- or LPS-activated DC on MCF-7, HBL-100, MDA-MB-231, or MDA-MB-415 breast cancer cell lines. Figure 3 represents the growth inhibition of tumor cells by LPS-activated DC. Similar results were obtained with DC preparation after activation with IFN- γ and IL-15 (data not shown). These results demonstrate that TNF- α produced by activated DC may contribute to growth inhibition of breast cancer cell lines.

The identity of TNF- α -mediated growth inhibition by activated DC was further supported by increased growth inhibition of breast cancer cells by cell-free culture supernatant derived from DC after LPS treatment. Treatment of this supernatant (pretreated with polymyxin B to inactivate the LPS) with anti-human TNF- α and not by anti-human IL-12 significantly inhibits the tumor cell growth. The breast cancer cell and the DC-derived supernatant were incubated in the same way before being pulsed for determination of proliferation of individual tumor cell lines (Fig. 4). These data suggest that TNF- α derived from activated DC could act as a potential antitumor molecule, which significantly inhibits the growth of breast cancer cells. Supernatants generated from DC after treatment with IFN- γ or IL-15 were also studied after neutralizing the endogenous IFN- γ or IL-15 with mAb specific to indicated cytokine before used for tumor growth inhibition study (data not shown).

The expression of intracellular TNF- α has been documented in DC after stimulation with IFN- γ , IL-15, or LPS. TNF- α also induces a low level of TNF- α in DC. Figure 5 represents intracellular TNF- α production in DC stimulated with media alone or indicated cytokines or LPS. The light-colored histogram represents the isotype control, and the overlaid, dark-colored histogram represents the staining with TNF- α .

Cytotoxicity activity of DC on breast cancer cells

In our preliminary experiment, we observed that several breast cancer lines were not sensitive to the human DC in a standard 4-h ^{51}Cr release assay (data not shown). However, if the coculture of activated DC and tumor targets extended beyond 10 h or more, breast tumor targets are susceptible to the DC-mediated lysis. Breast cancer cells incubated with DC pretreated with IFN- γ , IL-15, or LPS show enhanced lysis compared with DC alone or DC treated with GM-CSF. Lysis of MCF-7 cell lines was augmented from <5% by DC alone to as much as 30% in the presence of DC pretreated with LPS (Fig. 6A). Similar results were observed with three other cell lines

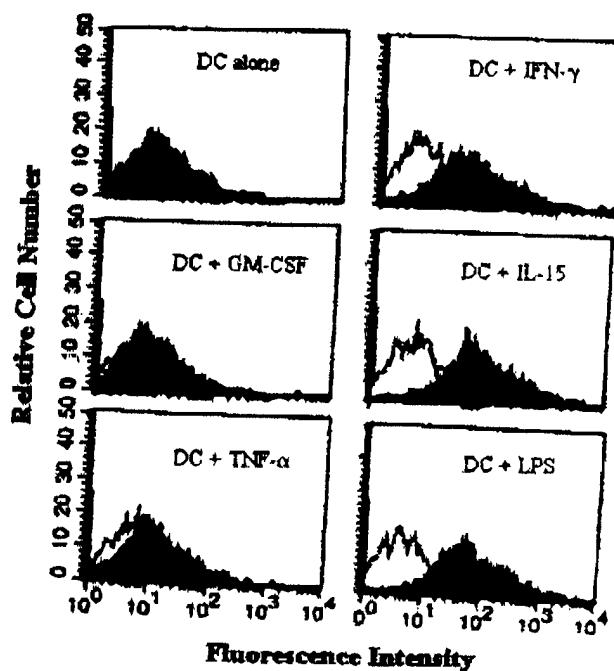


Fig. 5. Intracellular staining of TNF- α in DC after treatment with cytokines or LPS. Blood DC were cultured with media alone or media supplemented with GM-CSF, TNF- α , IFN- γ , IL-15, or LPS for 24 h. The DC were fixed with 1% p-HCHO (para-formaldehyde) for 5 min at room temperature followed by washing, and fixed with α -gluco pyranoside for 5 min at room temperature. The fixed, permeabilized cells were stained with mouse anti-human TNF- α for 45 min on ice and analyzed by fluorescein-activated cell sorter (FACS) analysis. The light-colored histogram represents the isotype control, and the overlaid, dark histogram represents the TNF- α staining in indicated treatment. Representative of four experiments is shown here.

tested (HBL-100, MDA-MB-231, and MDA-MB-415). No significant enhancement of lysis was observed on tumor cells after coculture with TNF- α - or GM-CSF-activated DC (Fig. 6, A–D). DC-mediated cytotoxicity of breast cancer cell lines appears to be specific, as the LCL and MRC-5 as well as three other breast cancer cell lines showed no significant lysis by unstimulated as well as by activated DC (Table 1). The spontaneous release varies from 10–18% among the tumor targets tested.

Induction of apoptosis of breast cancer cell lines by activated DC

The results presented in earlier sections clearly indicate that cytokine-treated DC have enhanced cytotoxicity against several breast cancer lines. However, this experiment does not differentiate necrotic versus apoptotic cell death. To determine the DC-mediated induction of apoptotic cell death of the target cells, the binding of Annexin V to the breast cancer cells was measured. Light scatter characteristics were used to distinguish the tumor cells from the DC, such that only the breast cancer cells were counted in the analysis. After 8 h of incubation, only those breast cancer cells incubated with unstimulated DC or cytokine-stimulated DC (E:T, 10:1) were positive for Annexin V binding. In breast cancer cell line MCF-7, increased Annexin V-positive cells were observed after treat-

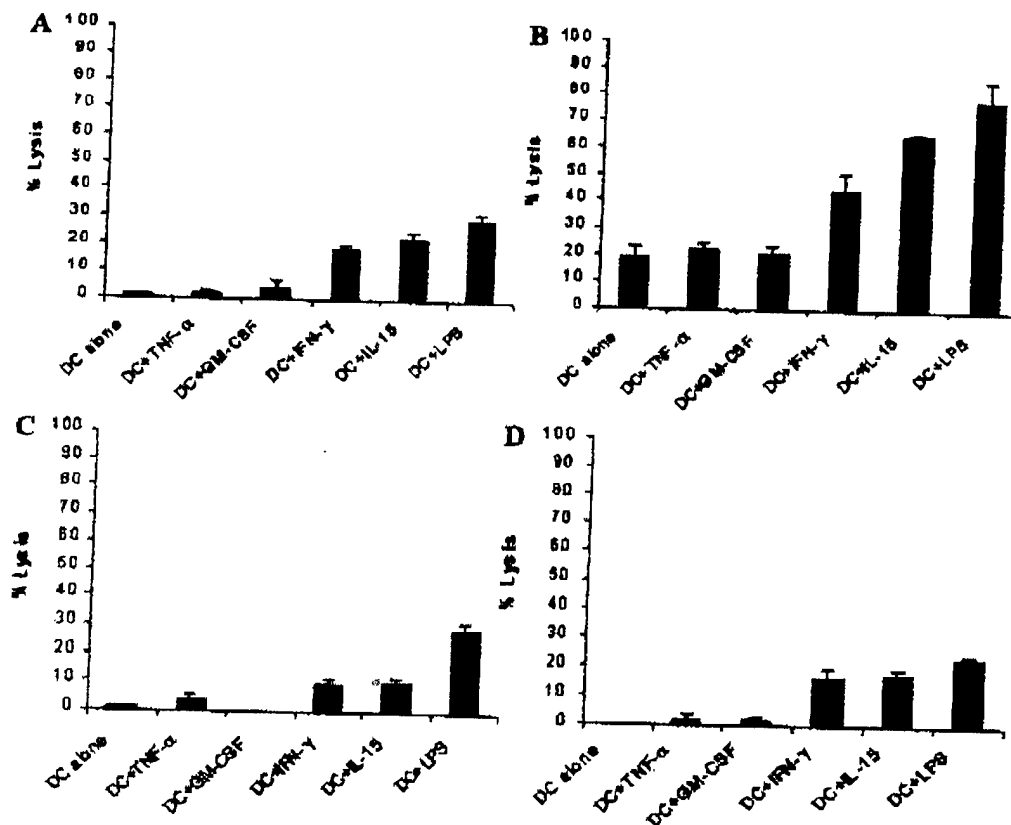


Fig. 6. Cytolytic activity of human DC against breast cancer cell lines. DC were cultured alone or in the presence of recombinant human TNF- α , recombinant human GM-CSF, recombinant human IFN- γ , recombinant human IL-15, or LPS for 24 h at 37°C, 5% CO₂, in 96-well plates. After extensive washing, 5×10^3 ⁵¹Cr-labeled MCF-7 (A), HBL-100 (B), MDA-MB-231 (C), or MDA-MB-415 (D) was added into wells at an E:T ratio of 10:1 and incubated for 18 h at 37°C, 5% CO₂. The supernatants were collected and released; ⁵¹Cr-labeled Cr was measured in a radioactive counter. The results are presented as the mean \pm SD of triplicate wells. The experiment was repeated five times with unrelated donors and produced similar results.

ment with LPS-treated DC (17.30%) compared with treatment with DC alone (4.8%). Treatment with IFN- γ - or IL-15-treated DC also shows higher Annexin V-positive cells compared with the treatment with unactivated DC (Fig. 7A). Similar results were observed in other cell lines tested. The differences in Annexin positivity among the tumor cell lines could be attributed to the wide biological differences in the tumor cell line

and unknown DC-tumor interaction. HBL-100 was particularly susceptible to DC-mediated apoptosis. The percent apoptosis was found to be higher in this cell line compared with others tested (Fig. 7B). This result was also tallied with the increased susceptibility of HBL-100 to DC-mediated growth inhibition and cytotoxicity. Two other cell lines also showed enhanced apoptosis in the presence of activated DC (Fig. 7, C and D).

TABLE 1. Tumoricidal Activity of Cytokine-Stimulated, Peripheral Blood-derived DC

Target cell	# Donors tested	DC alone	DC + TNF- α	DC + GM-CSF	DC + IFN- γ	DC + IL-15	DC + LPS
MCF-7	5	1.47 \pm 2.0	2.35 \pm 1.8	0.8 \pm 0.5	10.32 \pm 1.4	32.64 \pm 7.0	29.14 \pm 5.0
HBL-100	5	18.84 \pm 2.3	18.54 \pm 3.7	16.56 \pm 3.0	40.12 \pm 3.2	57.37 \pm 4.2	66.82 \pm 6.2
MDA-MB-231	5	5.70 \pm 4.5	5.08 \pm 4.1	5.54 \pm 4.4	14.93 \pm 2.3	24.86 \pm 4.8	47.25 \pm 3.1
MDA-MB-415	5	0 \pm 0	2.05 \pm 1.8	2.27 \pm 2.4	15.93 \pm 2.9	21.70 \pm 4.8	26.85 \pm 4.9
BT-20	2	0.98 \pm 0.5	1.12 \pm 0.1	1.89 \pm 1.7	2.13 \pm 0.8	7.90 \pm 4.9	6.69 \pm 1.6
MDA-MB-175	2	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	1.70 \pm 1.3
BT-474	4	0 \pm 0	0 \pm 0	0 \pm 0	4.22 \pm 2.4	3.47 \pm 2.8	13.23 \pm 4.2
MRC-5	3	0 \pm 0	0.25 \pm 0.1	0 \pm 0	0.28 \pm 0.1	0.9 \pm 0.04	0 \pm 0
LCL	3	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	1.61 \pm 0.5	3.76 \pm 1.8
LCL	3	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	5.74 \pm 2.3

Means were calculated from experiments performed with DC incubated with media alone or indicated cytokines and stimulating agent before used as an effector in ⁵¹Cr release assay. Mean percent-specific lysis \pm SD at 10:1 DC: target cell ratio.

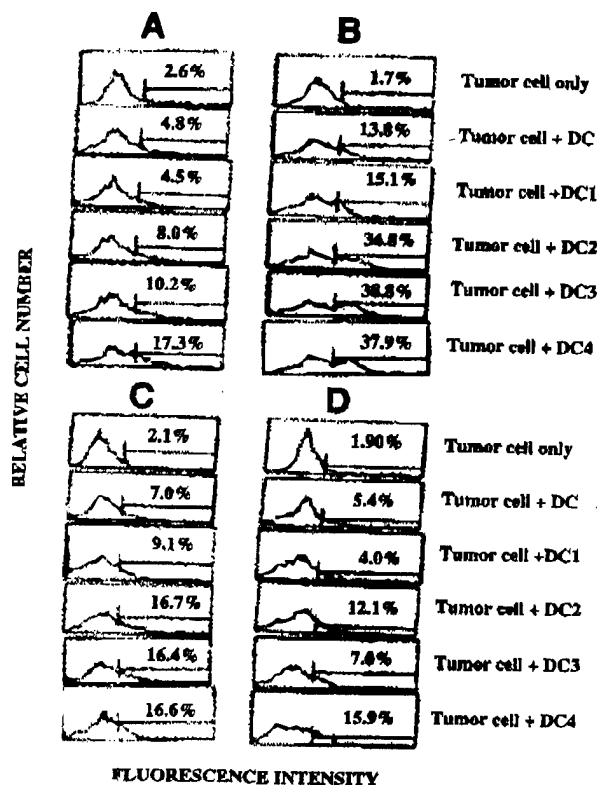


Fig. 7. Breast tumor targets undergo enhanced apoptotic cell death by activated DC. Tumor cell targets undergo significant apoptotic cell death when cultured with IFN- γ , IL-15, or LPS-stimulated DC as determined by phosphatidyl serine externalization. MCF-7 (A), HBL-100 (B), MDA-MB-231 (C), or MDA-MB-415 (D) tumor cells were cultured for 8 h in complete medium alone, in the presence of unstimulated DC, or with GM-CSF (DC1), IFN- γ (DC2), IL-15 (DC3), or LPS-stimulated DC (DC4) at an E:T ratio 10:1. Cells were then stained with FITC-Annexin V and analyzed by flow cytometry. The percentage of Annexin V-positive tumor cells is indicated for each condition. Histograms represent 10⁴-gated tumor cells. The experiment was repeated two times using two different donors with identical results.

Prolonged incubation of breast cancer cells with DC (unactivated and activated) leads to an increased percentage of Annexin V binding. Figure 8, A and B, represents Annexin V and Annexin V/PI staining of HBL-100 after 24 h incubation with unactivated and activated DC. This result indicates that prolonged incubation with DC leads to significant killing of tumor targets. The other three cell lines, MCF-7, MDA-MB-231, and MDA-MB-415, also showed similar results (data not shown). The apoptosis-inducing, tumoricidal activity of the cytokine-stimulated DC was seen with DC from multiple donors and tumor cell targets. These results demonstrate that human peripheral blood DC can kill breast cancer cell lines by inducing apoptosis.

DISCUSSION

DC have been thought to play an important role in tumor-immune surveillance as well as eradication. A direct correlation between a better prognosis and the number of DC adjacent

to the tumor has been documented in several cancers such as adenocarcinoma of colon and lung, thyroid and prostate cancer, and gastric carcinoma [3, 22, 23]. DC have also been reported to be functionally impaired in tumor-bearing animals and patients [16, 24], and a sharp decline in the recruitment of functionally mature DC has been reported in many cancers [3]. The antigen-presenting capacity of DC and consequent T cell activation against the tumors have been well documented [3, 25]. In addition, there have been reports suggesting a direct role of DC as effector cells against different tumor targets [19, 21]. This study was aimed at defining the mechanism of direct effector function of DC against breast cancer.

In this study, DC derived from the peripheral blood were used. DC demonstrated typical phenotypes including CD1a, CD11c, and CD83. There was no contamination (<2%) with T cells (CD3), B cells (CD19), and natural killer cells (CD56) in the DC population used, indicating that the effector function presented in this study is mediated by DC. The expression of mature DC marker CD83 in our DC preparation was also supported by the similar observation made by Fanger et al. [19]. Our results demonstrate that activated human peripheral blood DC have enhanced cytostatic as well as a cytotoxic role against the breast tumor cell lines, which are initiated by the apoptosis of tumor cells. The tumor growth inhibition by DC was significantly enhanced by pretreatment of DC with IFN- γ , IL-15, or LPS. Conversely, pretreatment with TNF- α or GM-CSF had no significant effect (Fig. 2). We have observed that DC from all the donors have clear cytotoxic and cytostatic potential against the tumor cells after activation, although the degree of effector function varies among the donors and the tumor lines tested. The differential sensitivity of tumor cells to DC indicates the complex nature of DC-tumor interactions as well as heterogeneity of the individual tumor line. LPS-induced DC activation and its antitumor function are partially mediated through the participation of TNF- α and not by IL-12 or IFN- γ (Fig. 3). Similar observation was made with IFN- γ - or IL-15-activated DC (data not shown). Cell-free culture supernatant of LPS-activated DC also showed antitumor property, which is found to be inhibited by the pretreatment of supernatant with anti-TNF- α and not by anti-IL-12 (Fig. 4). As DC preparation from peripheral blood was absolutely free from monocytes, induction of TNF- α in DC is possibly mediated through the participation of Toll-like receptor by LPS treatment [26]. This observation is in agreement with the results by Chapoval et al. [21], where they induced TNF- α production in monocyte-derived DC after LPS treatment for the antitumor immunity. Activated DC from several donors is also cytotoxic to a number of breast cancer cell lines (Fig. 6, Table 1). There was no significant cytotoxicity against the B-LCL or the MRC-5 fibroblast line (Table 1).

Another important finding is that breast cancer cell lines cultured with activated DC underwent apoptosis. Apoptosis was noted as early as 8 h, indicating its role in breast cancer cell growth inhibition as well as lysis noted in our study. The degree of apoptosis was further enhanced upon longer incubation of DC and tumor cells (Fig. 8). It has been shown that tumor-derived apoptotic bodies can be taken up, processed, and presented to CD8⁺ T cells [4, 5]. The result presented in

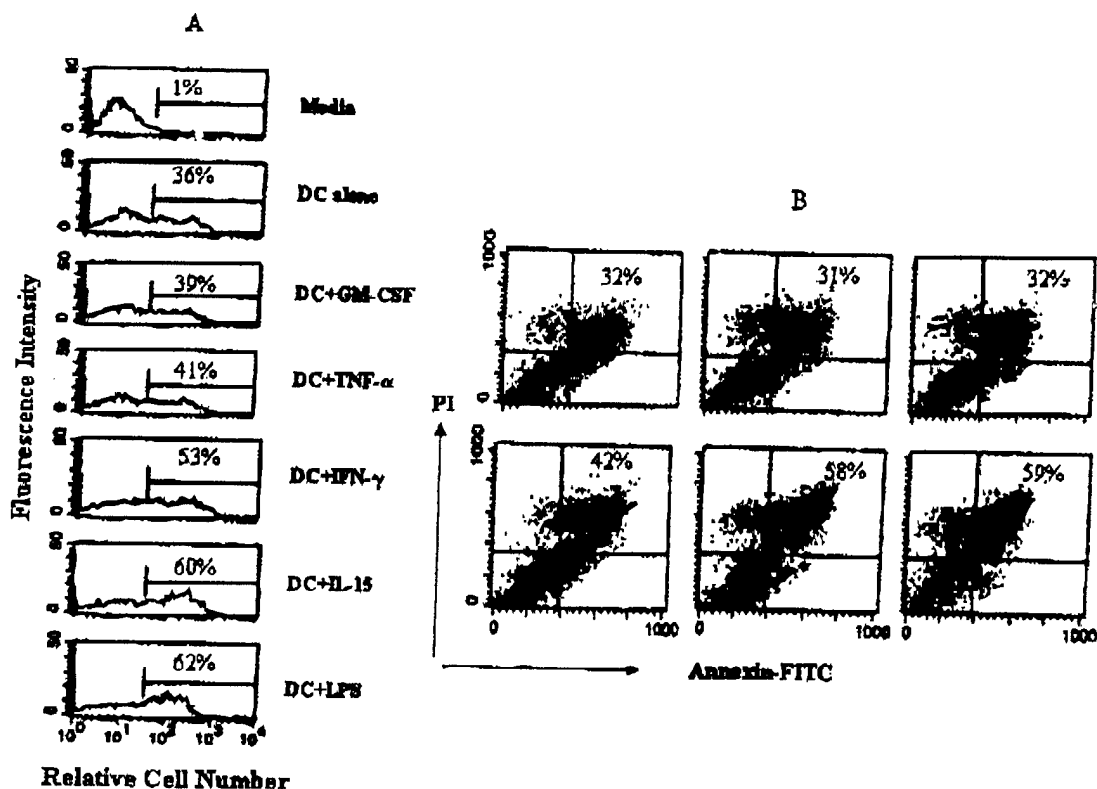


Fig. 8. Enhanced apoptotic cell death in tumor cells by DC upon longer incubation. Similar to Figure 7, the tumor cell HBL-100 was cultured with unactivated or activated DC for 24 h at an E:T ratio of 10:1. (A) The histogram presentation of Annexin V-positive cells upon treatment with unactivated or activated DC. (B) The double staining of the tumor cells with Annexin V and PI for demonstration of apoptotic as well as dead cells. Light scatter characteristics were used to distinguish the tumor cells from the DC, such that only the tumor cells were counted in the analysis. The experiment was repeated two times with similar results.

our study strongly suggests that DC may play an important role in this process.

In summary, the results presented herein indicate an important role of DC in host resistance against breast cancer. Peripheral blood DC induced apoptosis of several breast cancer cell lines and resulted in growth inhibition as well as lysis of breast cancer cells. These proactive roles of DC strongly suggest an important regulatory effector function for DC in the tumor microenvironment, which could prevent growth and metastasis of breast cancer. Taken together, we propose that drug-induced mobilization of DC in and around tumor sites may have a beneficial effect against the progression of the disease by the direct effector functions reported in this communication as well as the well-characterized tumor antigen presentation capability of DC.

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**Identification of HLA-A3-Restricted CD8+ T Cell Epitopes Derived from
Mammaglobin-A, a Tumor-Associated Antigen of Human Breast Cancer**

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Key words: Breast cancer, CD8+ T cells, HLA-A3, mammaglobin-A.

Abbreviations: CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunospot; T2.A3,
HLA-A3-transfected T2 cells.

Category: Tumor immunology.

Abstract

Mammaglobin-A is highly over-expressed in breast cancer cell lines and primary breast tumors. This pattern of expression is restricted to mammary epithelium and metastatic breast tumors. Thus, mammaglobin-A-specific T cell immune responses may provide an important approach for the design of breast cancer-specific immunotherapy. The purpose of this study was to define the T cell-mediated immune response to mammaglobin-A. We determined that the frequency of mammaglobin-A-reactive CD4⁺ and CD8⁺ T cells in breast cancer patients is significantly higher than that observed in healthy female controls using limiting dilution analyses ($P=0.026$ and $P=0.02$, respectively). Then, we identified eight mammaglobin-A-derived 9-mer peptides with the highest binding affinity for the HLA-A3 molecule (Mam-A3.1-8) using a computer-assisted analysis of the mammaglobin-A protein sequence. Subsequently, we determined that CD8⁺ T cells from breast cancer patients reacted to peptides Mam-A3.1 (23-31, PLEENVISK), Mam-A3.3 (2-10, KLLMVLMLA), Mam-A3.4 (55-63, TTNAIDELK), and Mam-A3.8 (58-66, AIDELKECF) using an IFN- γ ELISPOT assay. A CD8⁺ T cell line generated *in vitro* against HLA-A*0301-transfected TAP-deficient T2 cells loaded with these peptides showed significant cytotoxic activity against the Mam-A3.1 peptide. This CD8⁺ T cell line showed a significant HLA-A3-restricted cytotoxic activity against mammaglobin-A-positive but not mammaglobin-A-negative breast cancer cells. In summary, this study identified four HLA-A3-restricted mammaglobin-A-derived epitopes naturally expressed by breast cancer cells, indicating the immunotherapeutic potential of this novel antigen for the treatment and prevention of breast cancer.

Introduction

The evolution of normal breast tissue to breast cancer involves multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (1). To date most of the studies have concentrated on the MAGE and Erb-b2 families of tumor-associated genes in a variety of cancers (2-6). With the exception of the Her-2/neu and BRCA-1/2 oncogenes, there is only limited information available regarding the involvement of other genes in breast cancer (7;8). The mammaglobin-A gene was first identified using a differential screening approach directed at the isolation of novel human breast cancer-associated gene (9;10). The human mammaglobin-A gene is localized in chromosome 11q13 and encodes a 10 kDa glycoprotein that is distantly related to a family of epithelial secretory proteins that includes rat estramustine-binding protein/prostatein and human Clara cell protein (CC10/uteroglobulin) (11).

Mammaglobin-A has several properties that identify it as a clinically relevant breast cancer-associated marker (12-14). Unlike other genes over-expressed in breast cancer such as Erb/b2 and cyclin (15;16), the over-expression of mammaglobin-A seems to be breast cancer-specific (17). It is noteworthy that mammaglobin-A is significantly over-expressed in human breast cancer cell lines and primary human breast tumors (10;11;17). In a preliminary survey, about 60% of breast cancer cell lines and 62% of metastatic breast cancer tumors exhibited high levels of mammaglobin-A mRNA expression (10). More recently, it was observed that about 80% of primary breast cancer tumors have significantly high levels of mammaglobin-A expression at the protein level (17). Interestingly, staining was equally frequent among well differentiated (85%), moderately differentiated (91%), and poorly differentiated (72%) tumors. The over-expression of this gene may reflect a more cell specific alteration of the mammary epithelium rather than representing a general increased growth potential or mitotic rate (10).

Mammaglobin-A is largely restricted to mammary epithelium and breast cancer cells (10;11;17), as such, a clear understanding of the T cell-mediated immune response to this protein is of great importance in designing of specific immunotherapies against breast cancer. In this communication, we show that breast cancer patients have a significantly higher frequency of mammaglobin-A-reactive T cells as compared to normal individuals. We also identified four mammaglobin-A-derived epitopes presented in the context of HLA-A3 recognized by breast cancer patients. In addition, we show the *in vitro* generation of a peptide-specific CD8+ cytotoxic T lymphocyte (CTL) line with the ability to lyse breast cancer cells endogenously expressing mammaglobin-A.

Materials and Methods

Study subjects. Twelve patients with pathological diagnosis of breast carcinoma and ten healthy female controls were included in this study. Seven patients and six healthy female controls were included in the T cell quantification analysis using limiting dilution analysis. Five HLA-A3+ patients and four healthy female controls were included in the CD8+ CTL epitope analysis using an enzyme-linked immunospot (ELISPOT) assay. HLA typing of breast cancer patients and controls was performed by oligonucleotide sequence-specific primers that provided low to medium resolution for HLA-A genes (PEL-FREEZ, Brown Deer, WI) and high resolution for HLA-A3 genes (GenoVision Inc., Exton, PA) according to the manufacturer's instruction.

Cell lines. Six breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to the distributor's instructions (Table 1). HLA typing of these cell lines was performed as described and mammaglobin-A expression was determined by reverse transcriptase-polymerase chain reaction as previously described (10).

Peptides. Mammaglobin-A-derived peptides that bind the HLA-A3 molecule were identified using the HLA class I-binding prediction program from the National Institute of Health's Bioinformatics and Molecular Analysis Section at http://bimas.dcrt.nih.gov/molbio/hla_bind/ (18). Eight mammaglobin-A-derived 9-mer peptides with the highest binding score for the HLA-A3 molecule were used in our study (Table 2). Peptides were synthesized by Research Genetics, Inc. (Huntsville, AL). The purity of peptides was determined by high-performance liquid chromatography and mass spectrometry. The peptides were dissolved in DMSO at a concentration of 10 mg/ml and stored at -70°C until use.

HLA-A3 stabilization assay. The HLA-A3-binding ability of the peptides was confirmed by cell membrane stabilization of the HLA-A3 molecule in the TAP-deficient T2 cells transfected with the HLA-A*0301 gene (T2.A3 cells). Briefly, T2.A3 cells (1×10^6 /ml) were incubated in flat-bottom 96-well plates at 26°C in the presence of each peptide (80 µg/ml) in 200 µl of RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with AB+ normal human serum (10%, C-sis Diagnostics Inc., Mequon, WI), non-essential aminoacids (100 µM), L-glutamine (2 mM), HEPES buffer (25 mM), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml) (complete medium). Human β2 microglobulin (3 µg/ml, Sigma, St. Louis, MO) was also added to the cultures. After 18 hours, the T2.A3 cells were washed (3x) and the levels of HLA-A3 expression were determined by flow cytometric analysis. Briefly, the T2.A3 cells were incubated for 30 minutes at 4°C with the GAP.A3 anti-HLA-A3 monoclonal antibody (1:50, C-sis Diagnostics, Inc.) in PBS supplemented with BSA (2%), HEPES buffer (25 mM), and sodium azide (0.02%). The cells were then washed (3x) and incubated for 30 minutes at 4°C with FITC-conjugated goat anti-mouse IgG (1:50, Becton Dickinson, Franklin Lakes, NJ). The cells were then washed (3x), fixed in 1% paraformaldehyde, and used in a

single-color flow cytometric analysis in a FACScan flow cytometer (Becton Dickinson). T2.A3 cells cultured in the presence of the HLA-A3-binding ILRGSAHK influenza-derived peptide were used as a positive control (19). Results expressed as the mean fluorescence shift corresponded to the difference between the mean fluorescence obtained with the T2.A3 cells cultured in the presence of peptide (experimental) and the mean fluorescence obtained with empty T2.A3 cells (negative control). No mean fluorescence shift was observed with the MOPC-11 monoclonal antibody used as isotype control (data not shown).

Enzyme-linked immunospot assay. ELISPOT plates (Cellular Technologies, Cleveland OH) were coated with the M-700A-E anti-IFN- γ monoclonal antibody (5 μ g/ml, Endogen, Woburn, MA) at 4°C for 12 hours in 0.05 M carbonate-bicarbonate buffer (pH = 9.6). The plates were blocked with BSA (1%) for one hour and then washed (3x) with PBS. Subsequently, 3×10^5 peripheral blood mononuclear cells were cultured in triplicate wells in the antibody-coated plates in 200 μ l of complete medium in the presence of individual peptides (40 μ g/ml). Cells cultured in the presence of the ILRGSAHK influenza-derived peptide were used as positive control (19). Cells cultured in complete medium alone were used as negative control. After 48 hours, the plates were washed (3x) with PBS alone and with PBS supplemented with 0.5% Tween-20 (PBS-Tween). The plates were incubated at 4°C in the presence of the biotin-labeled M-701-B anti-IFN- γ monoclonal antibody (3 μ g/ml, Endogen) diluted in PBS-Tween supplemented with 1% BSA (PBS-Tween-BSA). After an overnight incubation, the plates were washed (3x) with PBS-Tween and incubated at room temperature in the presence of horseradish peroxidase-labeled Streptavidin (Endogen) diluted (1:2000) in PBS-Tween-BSA. After 2 hours, the plates were washed with PBS-Tween (2x) and with PBS alone (2x). The spots were developed by incubation in the presence of AEC (Sigma, 10 mg/ml in N,N-dimethyl formamide) freshly

diluted (1 ml in 30 ml) in 0.1 M sodium-acetate buffer (pH = 5.0). This AEC solution was filtered, mixed with 15 μ l of 30% hydrogen peroxide and added to the plates (200 μ l/well). After 10-20 minutes, the plates were washed (3x) with distilled water and air-dried. The resulting spots were counted on a computer assisted ELISPOT image analyzer (Cellular Technology). The number of spots observed in the negative control wells were subtracted from the number of spots observed in the experimental wells. Results are expressed as the mean number of IFN- γ -producing cells/ 1×10^6 cells.

Generation of peptide-specific CD8⁺ CTLs. After monocyte depletion, peripheral blood lymphocytes (2×10^6) were cultured in 2 ml of complete medium in 24-well plates in the presence of irradiated (6×10^3 rads) T2.A3 cells (1×10^6) loaded with peptides as described above. Human $\beta 2$ microglobulin (3 μ g/ml) and the CD28.2 anti-CD28 monoclonal antibody (500 ng/ml, PharMingen, San Diego, CA) were also added to the cultures. Low dose of recombinant human IL-2 (10 U/ ml, Chiron, Emeryville, CA) was added to the cultures after 24 hours. The cells (2×10^5) were restimulated every 7 days with peptide-loaded T2.A3 cells (1×10^6) in the presence of irradiated (2×10^3 rads) autologous peripheral blood lymphocytes (2×10^6) in 24-well plated in 2 ml of complete medium supplemented with IL-2, $\beta 2$ microglobulin, and anti-CD28. After three stimulations, the CD8⁺ T cells were purified by negative selection in a Mini Macs separation column using anti-CD4 MicroBeads (Miltenyi Biotec Inc., Auburn, CA). The T cells purified in this method were >95% CD8⁺ as determined by flow cytometric analysis (data not shown). The peptide-specific and mammaglobin-specific cytotoxic activity of the resulting CD8⁺ T cell line were analyzed 7 days after 3 to 5 stimulations.

Cytotoxic T lymphocyte activity assay. Peptide-loaded T2.A3 cells or breast cancer cells were labeled with ^{51}Cr (250 μ Ci, ICN Pharmaceuticals, Costa Mesa, CA) in 100 μ l of

complete medium. After one hour, 5×10^3 labeled cells were plated in triplicate cultures in round bottom 96-well plates in the presence of varying numbers of CD8+ T cells and incubated at 37°C for 18 hours. A 20-fold excess (1×10^5) of the HLA class I-negative K562 cell line was added to each well to inhibit non-specific lysis by NK cells. Control wells for determining spontaneous ^{51}Cr release contained labeled target cells alone. Maximal release was determined by adding Triton X-100 (1%) to the target cells. The percentage specific lysis was calculated as follows:
$$[(\text{experimental } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release}) / (\text{maximum } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release})] \times 100.$$

Epitope-specificity of breast cancer cell lysis by CD8+ CTLs was further determined in a cold target inhibition assay by analyzing the capacity of peptide-loaded unlabeled T2.A3 cells to block the lysis of breast cancer cells at an inhibitor to target ratio of 20:1.

For antibody blocking experiments, the target cells were incubated with the GAP-A3 anti-HLA-A3 monoclonal antibody (1:50), the BB7.2 anti-HLA-A2 monoclonal antibody (10 $\mu\text{g/ml}$), or the MOPC-11 isotype control monoclonal antibody (10 $\mu\text{g/ml}$) for 30 minutes before addition of the effector CD8+ T cells.

Limiting dilution analysis for CD8+ CTLs. The precursor frequency analysis of mammaglobin-reactive CD8+ CTLs was performed as previously described (20;21). Briefly, peripheral blood mononuclear cells were cultured at concentrations of 1×10^4 , 2×10^4 , and 4×10^4 cells/well (24 wells for each concentration) in round-bottom 96-well plates in complete medium supplemented with IL-2 (50 U/ml). After 7 days, 2×10^3 ^{51}Cr -labeled autologous dendritic cells pre-pulsed (50 $\mu\text{g/ml}$ /2 hours) with either recombinant mammaglobin-A or human serum albumin (Sigma) were added to each well and incubated for 18 hours. Specific lysis was calculated as described above. Cultures in the presence of dendritic cells pulsed with either

mammaglobin-A or albumin were considered positive when they showed 20% or higher specific lysis as compared to cultures in the presence of non-pulsed dendritic cells (negative control). The results are expressed as the reciprocal of the precursor frequency.

Limiting dilution analysis for CD4⁺ T cells. The precursor frequency analysis of mammaglobin-reactive CD4⁺ T cells was performed as previously described (21;22). Briefly peripheral blood mononuclear cells were cultured at concentrations of 1×10^4 , 2×10^4 , and 4×10^4 cells/well (24 wells for each concentration) in round-bottom 96-well plates in complete medium supplemented with IL-2 (50 U/ml). After 7 days, the cells were resuspended in fresh medium without IL-2. Then, 2×10^3 autologous dendritic cells were added to each well along with recombinant mammaglobin-A or albumin (1 μ g/well). After 48 hours, the cultures were pulsed with [³H]-thymidine (1 μ Ci/well). After 18 hours, [³H]-thymidine incorporation into DNA was determined by means of liquid scintillation counting. Cultures in the presence of mammaglobin-A or albumin were considered positive when their proliferative responses were 3-fold higher than the proliferative responses observed in cells cultured in the absence of proteins (negative control). The results are expressed as the reciprocal of the precursor frequency.

Statistical analysis. Statistical analysis of the limiting dilution analysis results was carried out by means of two-tailed unpaired-sample student's T test with the alpha set *a priori* at $P < 0.05$.

Results

Breast cancer patients have a higher frequency of mammaglobin-A-reactive T cells than healthy female controls. To determine whether mammaglobin-A-reactive T cells are expanded *in vivo* in breast cancer patients, we compared the precursor frequency of

mammaglobin-A-reactive T cells in seven breast cancer patients and six healthy female controls. As shown in Table 3, the frequency of mammaglobin-A-reactive CD8⁺ CTLs in peripheral blood of breast cancer patient ($2.4 \times 10^{-5} \pm 1.8 \times 10^{-5}$) was 9.6-fold higher than that observed in healthy female controls ($2.5 \times 10^{-6} \pm 1.8 \times 10^{-6}$) ($P=0.02$). No difference in the frequency of CD8⁺ CTLs reactive against albumin was observed between breast cancer patients ($3.7 \times 10^{-6} \pm 4.6 \times 10^{-6}$) and healthy female controls ($5.7 \times 10^{-6} \pm 1.6 \times 10^{-6}$) ($P=0.37$). As shown in Table 4, the frequency of mammaglobin-A-reactive CD4⁺ T cells in peripheral blood of breast cancer patient ($4.1 \times 10^{-5} \pm 3.1 \times 10^{-5}$) was 21.6-fold higher than that observed in healthy female controls ($1.9 \times 10^{-6} \pm 1.9 \times 10^{-6}$) ($P=0.026$). No difference in the frequency of CD4⁺ T cells reactive against albumin was observed between breast cancer patients ($4.6 \times 10^{-6} \pm 2.4 \times 10^{-6}$) and healthy female controls ($5.9 \times 10^{-6} \pm 4.1 \times 10^{-6}$) ($P=0.64$). Interestingly, one of the breast cancer patients (No. 5) did not show any CD8⁺ or CD4⁺ T cell reactivity against mammaglobin-A. This lack of reactivity may be due the fact that about 20% of breast cancer tumors do not express mammaglobin-A (17), however, this correlation could not be confirmed in this patient. Overall, these results indicate that mammaglobin-A-specific T cells are expanded *in vivo* in breast cancer patients.

Identification of HLA-A3-restricted CD8⁺ CTL epitopes derived from Mammaglobin-A. Using a computer-assisted analysis (18), we screened the mammaglobin-A protein for HLA-A3-binding 9-mer peptides and identified eight peptides with the highest binding probability to HLA-A3 (Table 2). The actual binding affinity of the peptides was determined by means of a HLA-A3 surface stabilization assay in T2.A3 cells. As shown in Figure 1, peptides Mam-A3.3, Mam-A3.5, Mam-A3.6, and Mam-A3.7 displayed a high affinity for the HLA-A3 molecule comparable to the one observed for the ILRGSAVHK influenza-derived peptide. On the contrary, peptides Mam-A3.1, Mam-A3.2, Mam-A3.4, and Mam-A3.8

displayed significantly lower affinity for the HLA-A3 molecule as compared to the ILRGSAHK influenza-derived peptide. The discrepancies between the predicted affinity of the peptides and their corresponding membrane stabilization ability of the HLA-A3 molecule may be due to the fact that the binding affinity of the peptide is determined by both the binding motifs as well as the 3-dimensional structure of the peptide in the MHC class I groove (23;24). Since it has been previously shown that the affinity of a given peptide to a MHC class I molecule does not necessarily correlate with its ability to generate CD8⁺ CTLs (25;26), we used all the peptides to examine the profile of CD8⁺ CTL reactivity in five HLA-A3⁺ breast cancer patients and four HLA-A3⁺ healthy female controls by means of an ELISPOT assay as described. As shown in Table 5, CD8⁺ CTL reactivity was observed against peptides Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8. Four patients (80%) showed CD8⁺ CTL reactivity against peptide Mam-A3.4, three patients (60%) showed CD8⁺ CTL reactivity against peptides Mam-A3.1 and Mam-A3.3, and two patients (40%) showed CD8⁺ CTL reactivity against peptide Mam-A3.8. It is noteworthy that patient No. 3 showed a markedly higher reactivity against peptide Mam-A3.3 as compared to the reactivity against the ILRGSAHK influenza-derived peptide. A reactivity comparable to the one observed against the ILRGSAHK influenza-derived peptide was detected against peptide Mam-A3.4 in patients No. 1 and 3. The magnitude of the reactivity against peptides Mam-A3.1 and Mam-A3.8 was markedly lower than the one detected against the ILRGSAHK influenza-derived peptide. No significant reactivity was observed against peptides Mam-A3.2, Mam-A3.5, Mam-A3.6, and Mam-A3.7. In addition, no reactivity was detected against any of the mammaglobin-A-derived peptides in the four healthy female controls included in this analysis (Table 5). Interestingly, these results show a differential recognition of these epitopes by different patients. As shown in Table 5, patients 2 and 3 showed a clear pattern

of recognition against peptides Mam-A3.3, Mam-A3.4, and Mam-A3.8. In contrast, patients 1, 4, and 5 showed a different pattern of recognition against peptides Mam-A3.1 and Mam-A3.3/4. These results clearly identify peptides Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 as CD8+ CTL epitopes presented by breast cancer tumors endogenously expressing mammaglobin-A.

Lysis of breast cancer cells by CD8+ CTLs generated *in vitro* against mammaglobin-A-derived peptides. To determine whether mammaglobin-A-reactive CD8+ CTLs were able to lyse breast cancer cells, we generated a CD8+ CTL line against the epitopes identified above. Toward this, T2.A3 cells were individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 peptides as described and pooled in equal numbers. Then, peripheral blood lymphocytes from an HLA-A3+ healthy female control were stimulated *in vitro* as described. In this regard, T2 cells have been shown to have comparable antigen-presenting efficiency to dendritic cells for the generation of CD8+ CTLs against self- and non-self proteins (26;27). After three weekly stimulations, the mammaglobin-A-restricted cytotoxic activity of the resulting CD8+ T cell line was evaluated against a panel of six different breast cancer cell lines (Table 1). As shown in Figure 2, the resulting CD8+ T cell line showed significant cytotoxic activity against the DU-4475 breast cancer cell line (HLA-A3+/mammaglobin-A+) but not against the HBL-100 (HLA-A3-/mammaglobin-A+), MDA-MB-415 (HLA-A3-/mammaglobin-A+), T-47D (HLA-A3+/mammaglobin-A-), and MCF-7 (HLA-A3-/mammaglobin-A-) breast cancer cell lines. No cytotoxic activity was observed against the NK-sensitive K562 cell line (data not shown).

It is interesting to note that this CD8+ CTL line did not show any cytotoxic activity against a breast cancer cell line that expresses both HLA-A3 and mammaglobin-A (AU-565,

Table 1). Since it has been shown that tumor cells selectively lose membrane expression of HLA class I molecules, the possibility existed that the lack of recognition of this breast cancer cell line by the anti-mammaglobin-A CD8⁺ CTL line was due to the lack of expression of the HLA-A3 molecule. To address this issue, we determined the levels of expression of both HLA-A2 and HLA-A3 molecules in the AU-565 cell line by flow cytometric analysis. As shown in Figure 3, the AU-565 cell line displays high levels of expression of the HLA-A2 molecule but no expression of the HLA-A3 molecule. These results indicate that the selective lack of membrane expression of the HLA-A3 molecule by the AU-565 cell line renders it resistant to the cytotoxic activity of the HLA-A3-restricted CD8⁺ CTL line generated *in vitro*. Overall, the results presented herein clearly demonstrate the HLA-A3-restriction and antigen-specificity of a CD8⁺ CTL line generated *in vitro* against the mammaglobin-A-derived epitopes.

Immunodominance of the CD8⁺ CTL response against mammaglobin-A-derived epitopes. To determine the epitope specificity of the resulting anti-mammaglobin-A CD8⁺ CTL line, T2.A3 cells were individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 peptides. Then, the peptide-loaded T2.A3 cells were used as targets in a standard CTL activity assay as described above. As shown in Figure 4, the resulting CD8⁺ CTL line showed significant cytotoxic activity against T2.A3 cells loaded with peptide Mam-A3.1 but not against T2.A3 cells loaded with peptides Mam-A3.3, Mam-A3.4 or Mam-A3.8. These data indicate the immunodominant nature of peptide Mam-A3.1 in this system. The epitope specificity and MHC-restriction of the cytotoxic activity of the CD8⁺ CTL line was confirmed in a cold target inhibition assay. As shown in Figure 5, lysis of the DU-4475 breast cancer cell line was blocked by the addition of T2.A3 cells loaded with the Mam-A3.1 peptide, whereas T2.A3 cells loaded with the Mam-A.3.3, Mam-A3.4, or Mam-A3.8 peptides showed no effect on the

cytotoxic activity against the DU-4475 breast cancer cell line. These results correlate with the computer-assisted analysis used to identify these peptides. As shown in Table 2, Mam-A3.1 has the highest binding probability to HLA-A3 among all the peptides tested.

Inhibition of the cytotoxic activity of the mammaglobin-A-reactive CD8+ CTL line by anti-HLA-3 antibodies. To confirm the presentation of the Mam-A3.1 peptide by the HLA-A3 molecule, the cytotoxic activity of the anti-mammaglobin-A CD8+ CTL line was determined in the presence of either HLA-A3 or HLA-A2 monoclonal antibodies. As shown in Figure 6, addition of the GAP-A3 anti-HLA-A3 monoclonal antibody inhibited the lysis of the DU-4475 breast cancer cell line whereas addition of the BB7.2 anti-HLA-A2 monoclonal antibody showed no effect on the cytotoxic activity against the DU-4475 breast cancer cell line. These results further confirmed the HLA-A3-restriction of the Mam-A3.1 peptide recognition by the anti-mammaglobin CD8+ CTL line.

Discussion

Previous studies have shown expression of the mammaglobin-A gene in about 80% of primary breast carcinomas (17). Based on the exclusive over-expression of mammaglobin-A by breast cancer cells, we investigated whether mammaglobin-A-reactive T cells were expanded in breast cancer patients. Furthermore, we identified mammaglobin derived CD8+ CTL epitopes, and demonstrated that it is possible to develop mammaglobin-A-reactive CD8+ CTL lines *in vitro* that recognize breast cancer cells naturally expressing mammaglobin-A derived peptides.

It is well-established that CD8+ CTLs are a critical component of host immunity to tumors due to their ability to recognize tumor-associated antigens in an MHC-restricted manner on several tumor cells (28;29). Therefore, definition of CD8+ CTL epitopes is an important area

for the development of breast cancer immunotherapies. Several studies have shown the *in vitro* generation of peptide-specific CD8⁺ CTLs against a variety of tumor-associated antigens such as Her-2/neu or MAGE (5;6). In this report, we identify another ubiquitously expressed breast cancer-associated antigen, mammaglobin-A, against which CD8⁺ CTLs can also be generated. Our study indicates that breast cancer patients have higher frequency of mammaglobin-A-reactive T cells as compared to healthy female controls (Tables 3 and 4). These results suggest that the over-expression this protein by breast cancer cells activates the expansion of normal low affinity mammaglobin-A-reactive T cells.

Using a computer-assisted analysis (18), we identified eight 9-mer peptides with the highest binding probability to HLA-A3. A similar approach has previously been used for the identification of HLA-A2-restricted CD8⁺ CTL epitopes derived from the MUC1 tumor antigen (30). Using an ELISPOT screening assay we were able to determine that four of these peptides were recognized by CD8⁺ CTLs from breast cancer patients *in vivo*. It is interesting that there seems to be different patterns of epitope recognition by different patients despite the fact that all the patients carried the HLA-A*0301 gene (Table 5). These results support the premise that these epitopes may be differentially expressed by breast cancer cells due to a differential enzyme cleavage of the mammaglobin-A protein in the proteosome (31). These data emphasize the importance of using several epitopes toward the design of effective immunotherapies for breast cancer.

To determine whether a CD8⁺ CTL line could be generated against mammaglobin-A with the ability to recognize breast cancer cells, we generated a CD8⁺ CTL line from a healthy female control using T2.A3 cells pulsed with the four mammaglobin-A-derived epitopes we identified by the ELISPOT assay (Table 5). The resulting cell line displayed a significant HLA-

A3-restricted cytotoxic activity against a mammaglobin-A+ DU-4475 breast cancer cell line (Figure 2). Further analysis demonstrated that this CD8+ CTL line recognized the Mam-A3.1 epitope demonstrating the immunodominant nature of this peptide in this system (Figures 4 and 5). This result may be due to the higher affinity for the HLA-A3 molecule of peptide Mam-A3.1 as compared to peptides Mam-A3.3 and Mam-A3.4 predicted by the computer analysis (Table 2). Interestingly, the Mam-A3.1 peptide was recognized by a lower number of CD8+ CTLs from breast cancer patients as compared to peptides Mam-A3.3 and Mam-A3.4 (Table 5). Therefore, it is likely that the Mam-A3.1 peptide may be presented in lower levels on breast tumors as compared to peptides Mam-A3.3 and Mam-A3.4 (32).

Our results show that mammaglobin-A peptide-specific CD8+ CTLs were also able to recognize breast cancer cells naturally expressing the mammaglobin-A protein in an epitope-specific and MHC-restricted manner (Figures 4 and 5). Our results also support previous reports on the ability of the T2 cell line as an effective antigen-presenting cell for the generation of CD8+ CTLs (26;27). The importance of this protocol is based in the fact that it does not depend on the maintenance and expansion of autologous dendritic cells for the generation and expansion of peptide-specific CD8+ CTLs against breast cancer. This technique could be useful for adoptive transfer of autologous tumor-specific CD8+ CTLs in breast cancer patients. Even though protocols for the maintenance and expansion of autologous dendritic cells generated from bone marrow-derived progenitors or peripheral blood monocytes have recently been established (3;6;30), they are more labor-intensive in comparison to the maintenance of a T2 cell line.

In summary, we have shown that mammaglobin-A-reactive T cells are expanded *in vivo* in breast cancer patients, we have identified four CD8+ CTL epitopes presented in the context of HLA-A3 from a breast cancer-specific antigen, mammaglobin-A, and we have shown that an *in*

vitro-generated CD8⁺ CTL line specific for the mammaglobin-A-derive Mam-A3.1 epitope has the ability to lyse breast cancer cells endogenously expressing this protein. These results shows that T2 cells pulsed with mammaglobin-A-derived peptides could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

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Table 1. Breast cancer cell lines

Breast cancer cell line	Tumor classification	HLA-A genotype¹	Mammaglobin expression²
DU-4475	Adenocarcinoma	HLA-A3,31	+
AU-565	Adenocarcinoma	HLA-A2,3	+
HBL-100	Adenocarcinoma	HLA-A1,2	+
MDA-MB-415	Adenocarcinoma	HLA-A30,33	+
T-47D	Adenocarcinoma	HLA-A3,26	—
MCF-7	Adenocarcinoma	HLA-A2,—	—

¹HLA typing of breast cancer cell lines was performed by sequence-specific primers that provided low to medium resolution for HLA-A genes (PEL-FREEZ) and high resolution for HLA-A3 genes (GenoVision Inc.). All the HLA-A3 cell lines carried the HLA-A*0301 allele.

²Mammaglobin expression was determined by reverse transcriptase-polymerase chain reaction as previously described (10).

Table 2. HLA-A3-binding peptides derived from mammaglobin-A

Peptide	Amino acid position	Peptide sequence	HLA-A3-binding score ¹
Mam-A3.1	23-31	PLENVISK	27.00
Mam-A3.2	31-39	KTINPQVSK	6.75
Mam-A3.3	02-10	KLLMVLMLA	4.05
Mam-A3.4	55-63	TTNAIDELK	1.50
Mam-A3.5	04-12	LMVLMLAAL	1.35
Mam-A3.6	66-74	FLNQTDETL	0.60
Mam-A3.7	07-15	LMLAALSQH	0.45
Mam-A3.8	58-66	AIDELKECF	0.30

¹Estimate of half time of HLA-A3-peptide disassociation based on the HLA class I-binding prediction program from the National Institute of Health's Bioinformatics & Molecular Analysis Section at http://bimas.dcrt.nih.gov/molbio/hla_bind/ (18).

Table 3. Frequency of mammaglobin-A-reactive CD8+ CTLs in peripheral blood of breast cancer patients and healthy female controls

Study Subjects	Mammaglobin-A-reactive ¹ CD8+ CTL frequency	Albumin-reactive ² CD8+ CTL frequency
Patients		
1	1.1x10 ⁻⁵	1.7x10 ⁻⁶
2	6.0x10 ⁻⁵	1.4x10 ⁻⁵
3	1.8x10 ⁻⁵	1.2x10 ⁻⁶
4	3.4x10 ⁻⁵	8.3x10 ⁻⁷
5	4.8x10 ⁻⁶	1.9x10 ⁻⁶
6	2.1x10 ⁻⁵	3.9x10 ⁻⁶
7	2.0x10 ⁻⁵	2.4x10 ⁻⁶
Mean ± SD:	2.4x10 ⁻⁵ ± 1.8x10 ⁻⁵	3.7x10 ⁻⁶ ± 4.6x10 ⁻⁶
Controls		
1	8.3x10 ⁻⁷	0 ³
2	NP ⁴	NP
3	5.0x10 ⁻⁶	4.6x10 ⁻⁶
4	3.6x10 ⁻⁶	6.8x10 ⁻⁶
5	1.1x10 ⁻⁶	0
6	1.9x10 ⁻⁶	0
Mean ± SD:	2.5x10 ⁻⁶ ± 1.8x10 ⁻⁶	5.7x10 ⁻⁶ ± 1.6x10 ⁻⁶

¹Frequency of mammaglobin-A-reactive T cells: Patients vs. Controls, P=0.02.

²Frequency of albumin-reactive T cells: Patients vs. Controls, P=0.37.

³Frequency in experimental cultures ≤ frequency in negative control cultures.

⁴NP: Not performed.

Table 4. Frequency of mammaglobin-A-reactive CD4+ T cells in peripheral blood of breast cancer patients and healthy female controls

Study Subjects	Mammaglobin-A-reactive ¹ CD4+ T cell frequency	Albumin-reactive ² CD4+ T cell frequency
Patients		
1	9.0×10^{-5}	4.8×10^{-6}
2	3.3×10^{-5}	4.8×10^{-6}
3	2.7×10^{-5}	2.1×10^{-6}
4	1.1×10^{-5}	3.9×10^{-6}
5	1.8×10^{-6}	8.9×10^{-6}
6	6.7×10^{-5}	3.0×10^{-6}
7	NP ³	NP
Mean \pm SD:	$4.1 \times 10^{-5} \pm 3.1 \times 10^{-5}$	$4.6 \times 10^{-6} \pm 2.4 \times 10^{-6}$
Controls		
1	NP	NP
2	4.2×10^{-7}	0 ⁴
3	1.7×10^{-6}	1.2×10^{-6}
4	5.1×10^{-6}	8.3×10^{-6}
5	1.4×10^{-6}	8.3×10^{-6}
6	8.3×10^{-7}	0
Mean \pm SD:	$1.9 \times 10^{-6} \pm 1.9 \times 10^{-6}$	$5.9 \times 10^{-6} \pm 4.1 \times 10^{-6}$

¹Frequency of mammaglobin-A-reactive T cells: Patients vs. Controls, P=0.026.

²Frequency of albumin-reactive T cells: Patients vs. Controls, P=0.64.

³NP: Not performed.

⁴Frequency in experimental cultures \leq frequency in negative control cultures.

Table 5. Frequency of CD8+ CTLs reactive to mammaglobin-A-derived peptides in peripheral blood of breast cancer patients

		HLA-A3-binding peptides								
		Mam-A3.1	Mam-A3.2	Mam-A3.3	Mam-A3.4	Mam-A3.5	Mam-A3.6	Mam-A3.7	Mam-A3.8	Influenza
Patients¹										
1	33 ²	0 ³	0	156	0	0	0	0	0	127
2	0	0	40	33	0	0	0	0	23	93
3	0	0	829	183	0	0	0	7	80	123
4	20	0	57	0	0	0	3	0	0	103
5	23	0	0	20	0	0	0	0	0	89
Controls¹										
1	0	0	3	0	0	0	0	0	3	83
2	0	0	0	0	0	0	0	0	0	96
3	0	0	7	3	0	0	7	0	0	109
4	3	0	0	0	0	0	0	0	0	76

¹All the patients and controls carried the HLA-A*0301 allele.

²Mean number of IFN- γ -producing cells/1x10⁶ cells (n=3 wells).

³Number of spots in experimental cultures \leq number of spots in negative control cultures.

Figure Legends

Figure 1. HLA-A3 stabilization by mammaglobin-A-derived peptides. The computer-assisted predicted HLA-A3-binding ability of the peptides was confirmed by cell membrane stabilization of the HLA-A3 molecule in the T2.A3 cells. T2.A3 cells were incubated in the presence of each peptide in complete medium. After 18 hours, the levels of HLA-A3 expression was determined by means of flow cytometric analysis. Results expressed as the mean fluorescence shift corresponded to the difference between the mean fluorescence obtained with T2.A3 cells cultured in the presence of peptide and the mean fluorescence obtained with empty T2.A3 cells.

Figure 2. Lysis of breast cancer cells by CD8⁺ CTLs generated *in vitro* against mammaglobin-A-derived peptides. A CD8⁺ CTL line was generated from an HLA-A3⁺ healthy female control by means of three weekly *in vitro* stimulations with pooled T2.A3 cells individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 peptides. The mammaglobin-A-specificity and HLA-A3-restriction of the resulting CD8⁺ T cell line was then evaluated against the DU-4475 (HLA-A3⁺/mammaglobin-A⁺), AU-565 (HLA-A3⁺/mammaglobin-A⁺) HBL-100 (HLA-A3⁻/mammaglobin-A⁺), MDA-MB-415 (HLA-A3⁻/mammaglobin-A⁺), T-47D (HLA-A3⁺/mammaglobin-A⁻), and MCF-7 (HLA-A3⁻/mammaglobin-A⁻) breast cancer cell lines.

Figure 3. Lack of HLA-A3 expression by the AU-565 breast cancer cell line. The level of HLA-A3 expression by the AU-565 breast cancer cell line was determined by flow cytometric analysis using the GAP-A3 monoclonal antibody and the control MOPC-11 monoclonal antibody followed by incubation with a FITC-conjugated goat anti-mouse IgG (A). The level of HLA-A2 expression by the AU-565 breast cancer cell line was used as internal

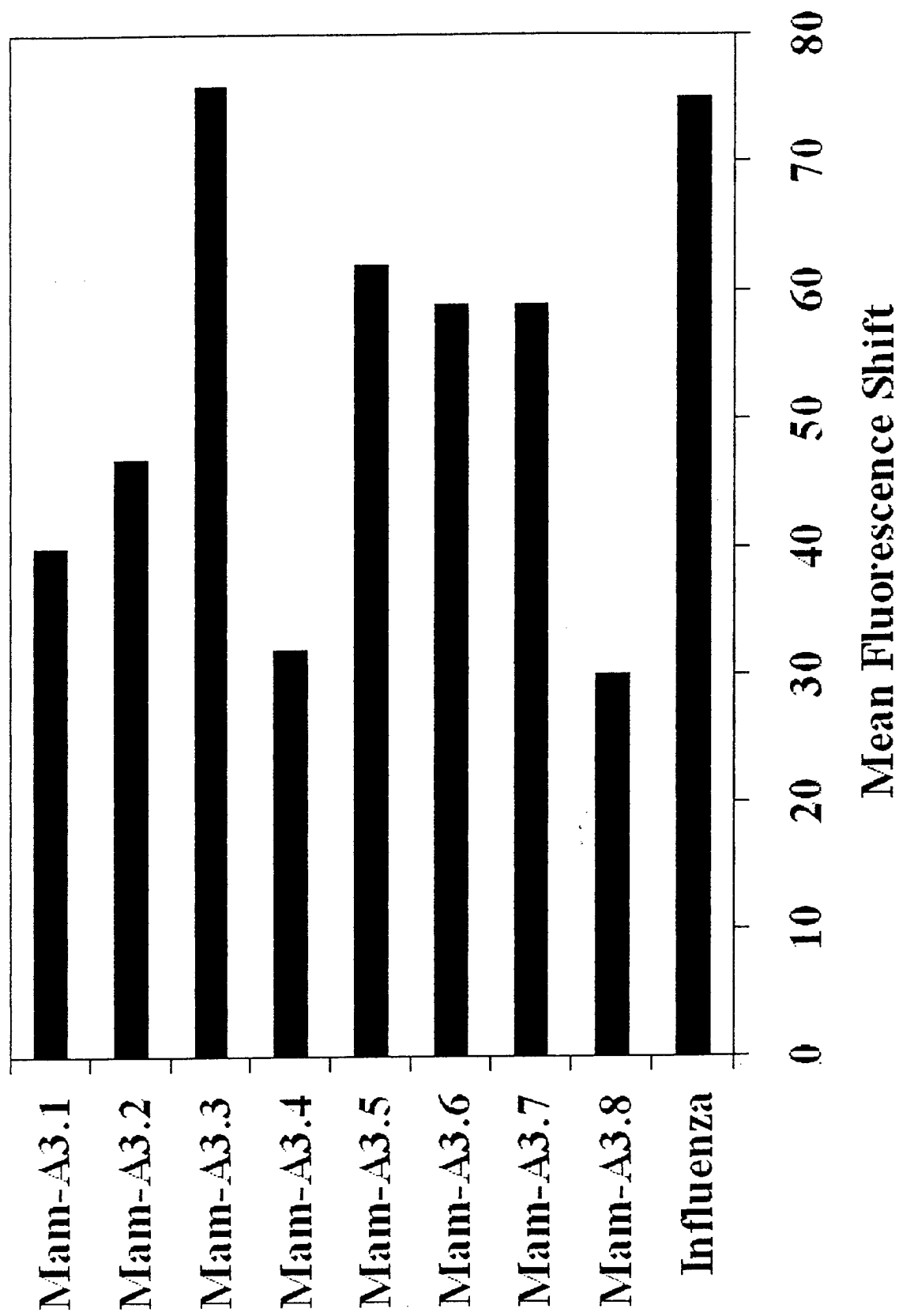
control and was determined by flow cytometric analysis using with the BB7.2 monoclonal antibody (B). The level of HLA-A3 expression by the DU-4475 breast cancer cell line was used as positive control and was determined by flow cytometric analysis using the GAP-A3 monoclonal antibody (C).

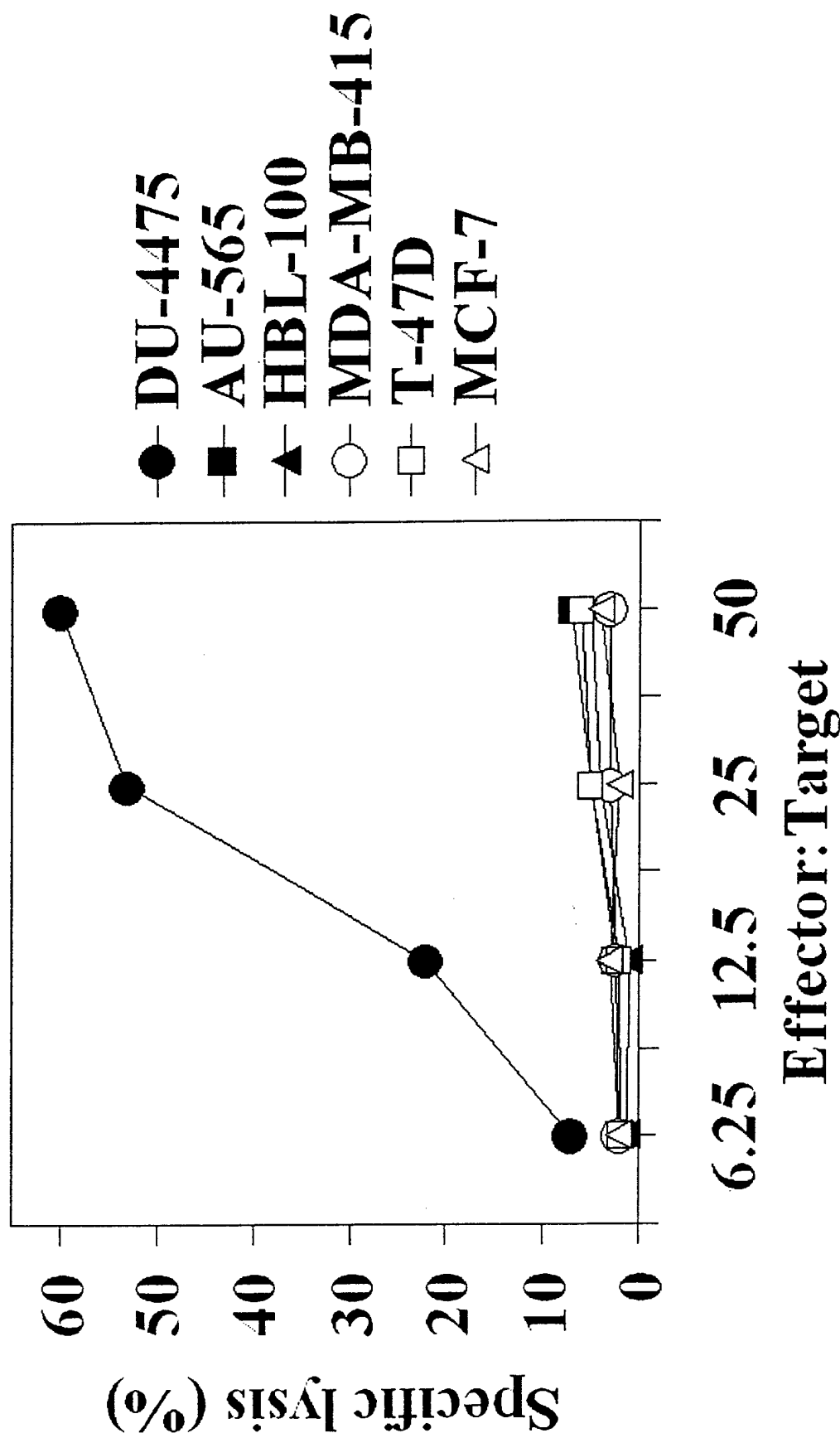
Figure 4. Immunodominance of the CD8+ CTL response against mammaglobin-A-derived peptides. A CD8+ CTL line was generated from an HLA-A3+ healthy female control by means of three weekly *in vitro* stimulations with pooled T2.A3 cells individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 peptides. The immunodominance of the resulting CD8+ T cell line was then evaluated against T2.A3 cells individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 peptides.

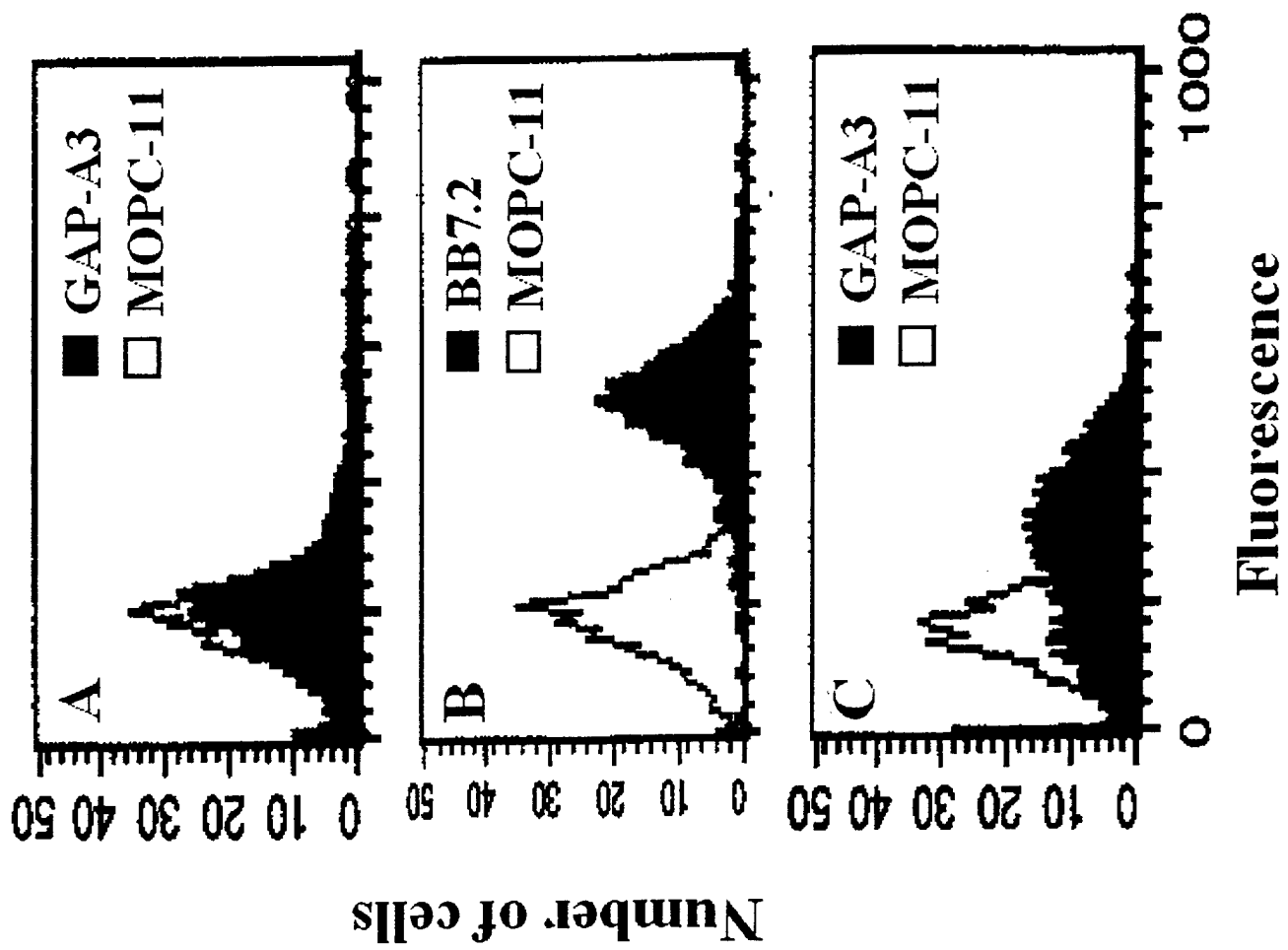
Figure 5. Recognition of the Mam-A3.1 peptide on DU-4475 breast cancer cells by CD8+ CTLs. A CD8+ CTL line was generated from an HLA-A3+ healthy female control by means of three weekly *in vitro* stimulations with pooled T2.A3 cells individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 peptides. The peptide-specificity of the resulting CD8+ T cell line was then evaluated against the DU-4475 breast cancer cell line (HLA-A3+/mammaglobin-A+) in the presence of a 20-fold excess of unlabeled T2.A3 cells individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, or Mam-A3.8 peptides. The assay was performed at an E:T ratio of 50:1.

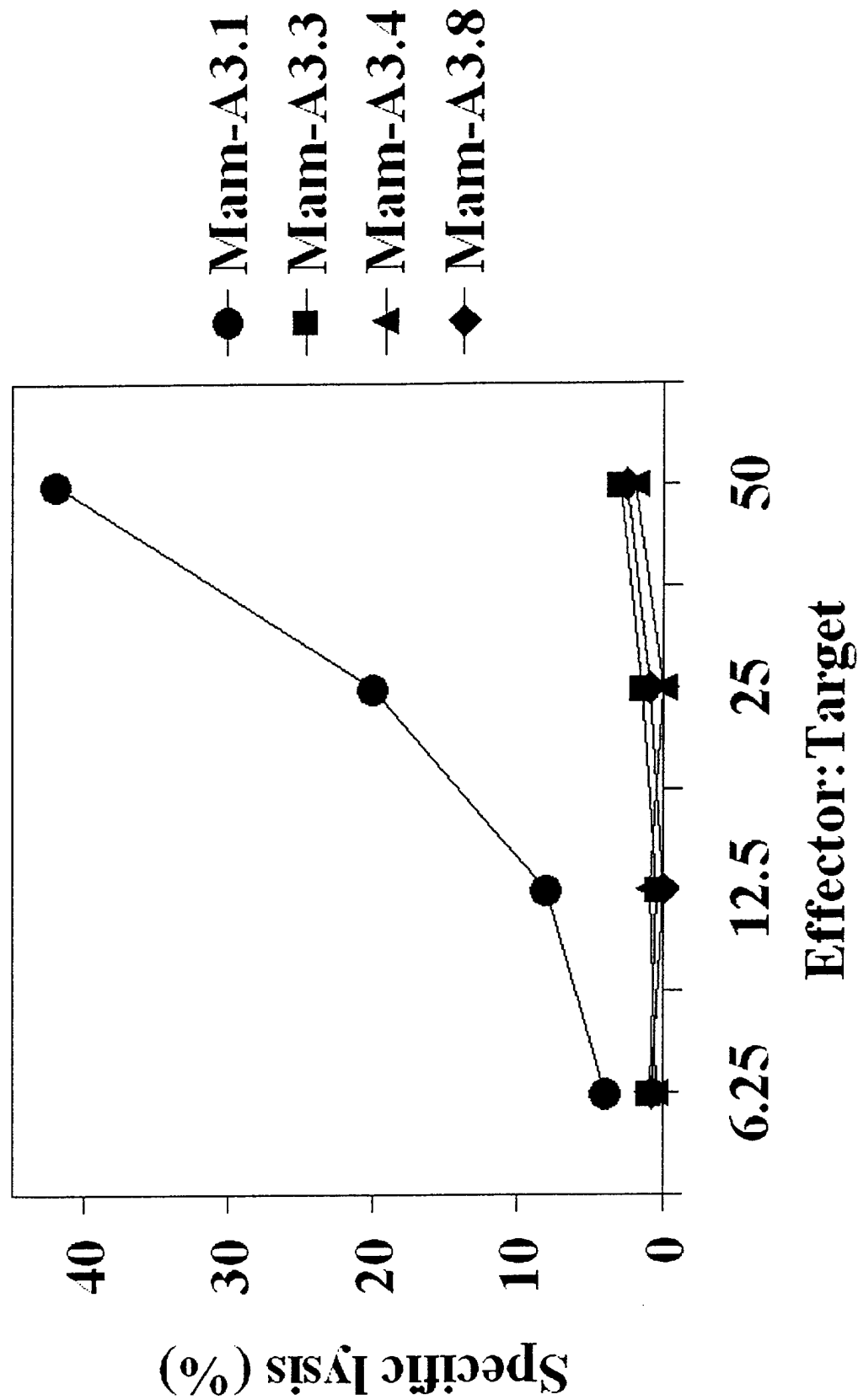
Figure 6. Inhibition of the cytotoxic activity of the mammaglobin-A-reactive CD8+ CTL line by anti-HLA-A3 antibodies. A CD8+ CTL line was generated from an HLA-A3+ healthy female control by means of three weekly *in vitro* stimulations with pooled T2.A3 cells individually loaded with the Mam-A3.1, Mam-A3.3, Mam-A3.4, and Mam-A3.8 peptides. The HLA class I-restriction of the resulting CD8+ T cell line was then evaluated against the DU-4475

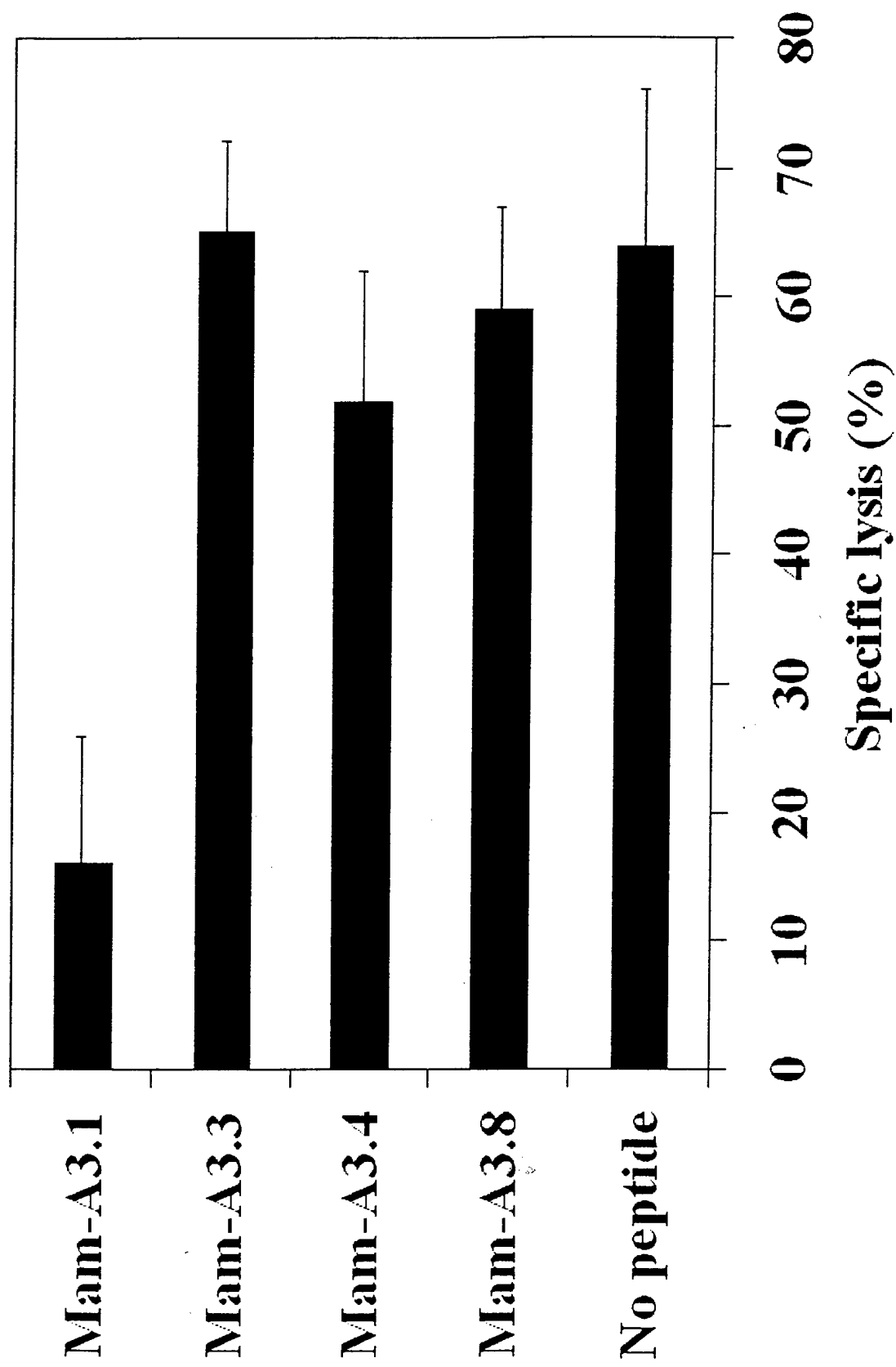
(HLA-A3+/mammaglobin-A+) in the presence of the GAP-A3 anti-HLA-A3 monoclonal antibody, the BB7.2 anti-HLA-A2 monoclonal antibody, or the MOPC-11 isotype control monoclonal antibody. The assay was performed at an E:T ratio of 50:1.

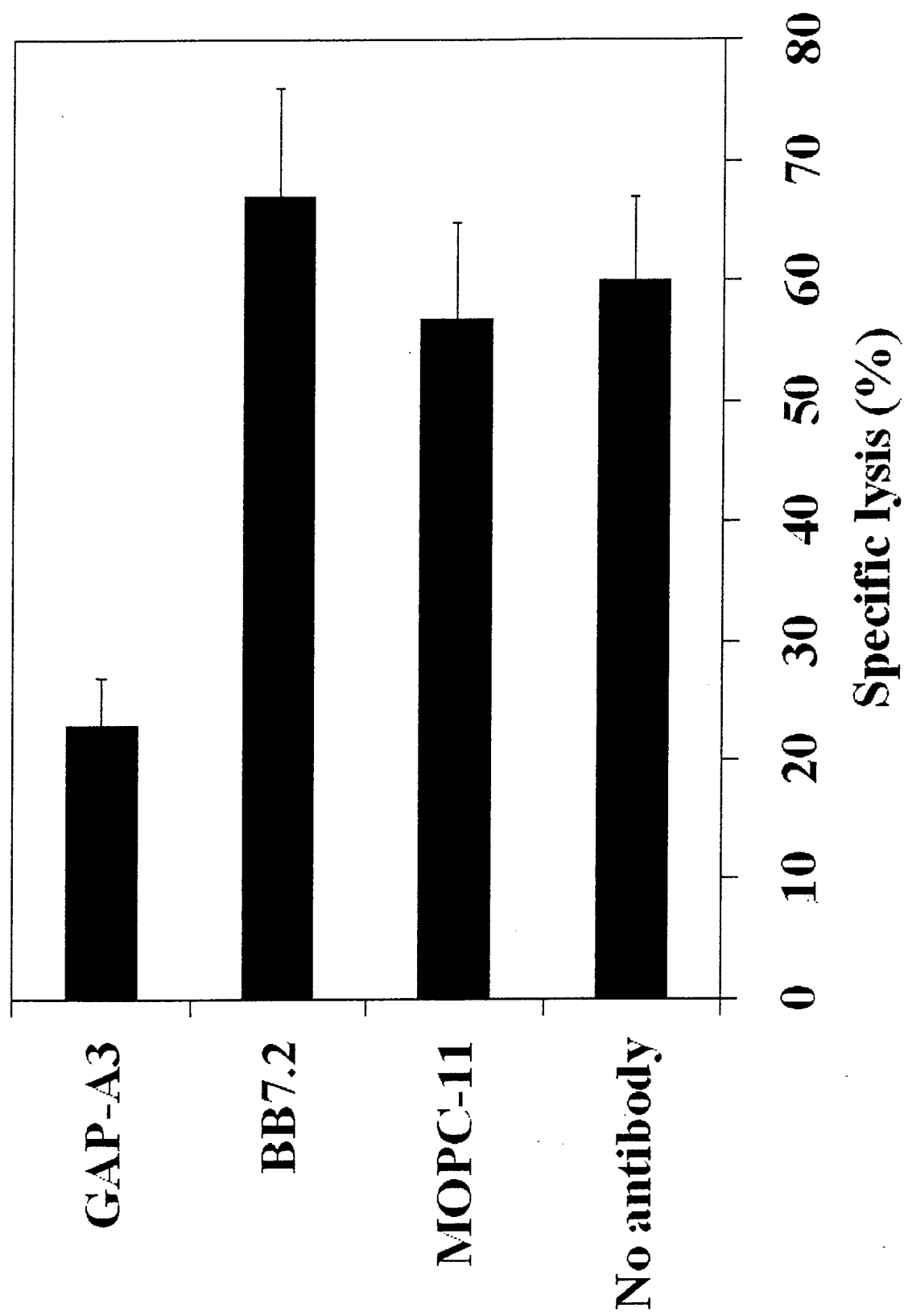












**Generation of CD8+ Cytotoxic T Lymphocytes against Breast Cancer Cells
by Stimulation with Mammaglobin-A-Pulsed Dendritic Cells**

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Summary

Mammaglobin-A is exclusively expressed by breast cancer cells. Thus, mammaglobin-A-specific T cell immune responses may be useful for the design of new breast cancer-specific immunotherapies. We show herein that CD8⁺ cytotoxic T lymphocytes generated against recombinant mammaglobin-A-pulsed dendritic cells displayed marked cytotoxic activity against mammaglobin-A-positive breast cancer cell lines. This study indicates the immunotherapeutic potential of this novel antigen for the treatment of breast cancer.

Key words: breast cancer-specific antigen, CD8⁺ cytotoxic T lymphocytes, mammaglobin-A

Introduction

The mammaglobin-A gene was first identified using a differential screening approach directed at the isolation of novel human breast cancer-associated gene (1;2). The human mammaglobin-A gene is localized in chromosome 11q13 and encodes a 10 kDa glycoprotein that is distantly related to a family of epithelial secretory proteins that includes rat estramustine-binding protein/prostatein and human Clara cell protein (CC10/uteroglobulin) 3). Mammaglobin-A has several properties that identify it as a clinically relevant breast cancer-associated marker (4-6). Unlike other genes over-expressed in breast cancer such as Erb/b2 and cyclin (7-8), the over-expression of mammaglobin-A seems to be breast cancer-specific (2;3;9). In a preliminary survey, about 60% of breast cancer cell lines and 62% of metastatic breast cancer tumors exhibited high levels of mammaglobin-A mRNA expression (2). More recently, it was observed that about 80% of primary breast cancer tumors have significantly high levels of mammaglobin-A expression at the protein level (9).

Mammaglobin-A is largely restricted to mammary epithelium and breast cancer cells (2,3,9), as such, a clear understanding of the T cell-mediated immune response to this protein is of great importance in designing of new immunotherapies against breast cancer. In this communication, we show the *in vitro* generation of a mammaglobin-A-specific CD8⁺ cytotoxic T lymphocyte (CTL) line with the ability to lyse breast cancer cells in a MHC class I-restricted and antigen-specific fashion.

Methods

Proteins. Recombinant human mammaglobin-A was produced with the PCI-neo expression vector (Promega, Madison, WI) containing the full-length mammaglobin-A cDNA. Human serum albumin was obtained from Sigma Chemical Company (St. Louis, MO).

Generation of dendritic cells. Peripheral blood mononuclear cells were seeded in 6-well plates (Costar, Cambridge, MA) at a concentration of 3×10^6 cells/well in 3 ml of RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with AB+ normal human serum (10%, C-Six Diagnostics, Germantown, WI), L-glutamine (2 mM), HEPES buffer (25 mM), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 μ g/ml) (complete medium). After 3 hours at 37°C, 5% CO₂, the non-adherent cells were removed, and the remaining adherent cells were cultured for 7 days in the presence of recombinant GM-CSF (1000 units/ml) and recombinant IL-4 (100 ng/ml) (10).

Generation of CD8+ CTLs. For CTL induction, 2×10^5 dendritic cells from an HLA-A2+ breast cancer patient were pulsed with mammaglobin-A (50 μ g/ml for 2 hours), washed and incubated with 5×10^5 autologous non-adherent peripheral blood lymphocytes in 96-well plates in complete medium for 7 days. Then, the cultures were re-stimulated with mammaglobin-A-pulsed dendritic cells in the presence of recombinant IL-2 (10 units/ml) for 5 days. After four stimulations, the CD8+ CTLs were purified by negative selection in a Mini Macs separation column using anti-CD4 and anti-CD56 monoclonal antibodies (BD PharMingen, San Diego, CA) followed by goat anti-mouse IgG-coated microBeads (Miltenyi Biotec Inc., Auburn, CA). The T cells purified in this method were >95% CD8+ as determined by flow cytometric analysis (data not shown).

Cytotoxic T lymphocyte activity assay. Breast cancer cells or mammaglobin-A-pulsed dendritic cells were labeled with 250 μCi of ^{51}Cr ($\text{Na}^{51}\text{CrO}_4$, ICN Pharmaceuticals, Costa Mesa, CA) in 100 μl of complete medium. After one hour, the labeled cells were plated in round bottom 96-well plates in triplicate cultures at a concentration of 5×10^3 cells/well and the effector CD8^+ CTLs were plated at a concentration of 2.5×10^5 cells/well (E:T ratio, 50:1). Then, the cultures were incubated at 37°C for 18 hours. For antibody blocking analysis, the target cells were incubated with the W6/32 anti-HLA class I or the KuIA2 anti-HLA-DR monoclonal antibodies (10 $\mu\text{g/ml}$) for 30 minutes before addition of the effector cells.

Results and Discussion

To analyze the antigen-specific cytotoxic activity of the CD8^+ CTL line generated against mammaglobin-A, we determined the ability of these CD8^+ CTLs to lyse autologous dendritic cells pulsed with this protein. As shown in Figure 1, this CD8^+ CTL line demonstrated antigen-specific killing of mammaglobin-A-pulsed dendritic cells. No lytic activity was observed with non-pulsed dendritic cells as well as albumin-pulsed dendritic cells. The lytic activity observed with the mammaglobin-A-pulsed dendritic cells was inhibited by the W6/32 monoclonal antibody (60% inhibition) but not by the KuIA2 monoclonal antibody (12% inhibition). These data demonstrate the antigen-specific cytotoxic activity by this CD8^+ CTL line.

To determine whether mammaglobin-A-reactive CD8^+ CTLs were able to lyse breast cancer cells, the cytotoxic activity of this CD8^+ CTL line was evaluated against a panel of six different breast cancer cell lines (Table 1). As shown in Figure 2, this CD8^+ CTL line showed significant cytotoxic activity against the HBL-100 and AU-565 cell lines (HLA-

A2+/mammaglobin-A+) but not against the MDA-MB-361 and MDA-MB-415 cell lines (HLA-A2-/mammaglobin-A+) as well as the MDA-MB-231 and MCF-7 cell lines (HLA-A2+/mammaglobin-A-). These results clearly demonstrate the ability of this CD8+ CTL line to recognize mammaglobin-A-derived epitopes on breast cancer cells.

It is well-established that CD8+ CTLs are a critical component of host immunity to tumors due to their ability to recognize tumor-associated antigens on several tumor cells (11;12). Therefore, definition of CD8+ CTL epitopes is an important area for the development of novel breast cancer immunotherapies. Several studies have shown the *in vitro* generation of peptide-specific CD8+ CTLs against a variety of tumor-associated antigens such as Her-2/neu or MAGE (13,14). In this report, we identify another ubiquitously expressed breast cancer-associated antigen, mammaglobin-A, against which CD8+ CTLs can also be generated.

Dendritic cells have been shown to prime naïve CD8+ CTL when they are pulsed either with peptides, proteins, or cell sonicates (15-18). To determine the feasibility to generate a CD8+ CTL line with the ability to recognize breast cancer cells, we use mammaglobin-A-pulsed dendritic cells. Our results indicate that mammaglobin-A-specific CD8+ CTLs have the capacity to lyse breast cancer cells. Thus, the results shown herein indicate the immunotherapeutic potential of mammaglobin-A for designing new immunotherapy protocols for the treatment of breast cancer.

Acknowledgements

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Table 1. Breast cancer cell lines

Breast Cancer Cell Lines	Tumor Classification	HLA-A Genotype¹	Mammaglobin Expression²
HLB-100	Adenocarcinoma	HLA-A1,2	+
AU-565	Adenocarcinoma	HLA-A2,3	+
MDA-MB-361	Adenocarcinoma	HLA-A1,24	+
MDA-MB-415	Adenocarcinoma	HLA-A30,33	+
MCF-7	Adenocarcinoma	HLA-A2,—	—
MDA-MB-231	Adenocarcinoma	HLA-A2, —	—

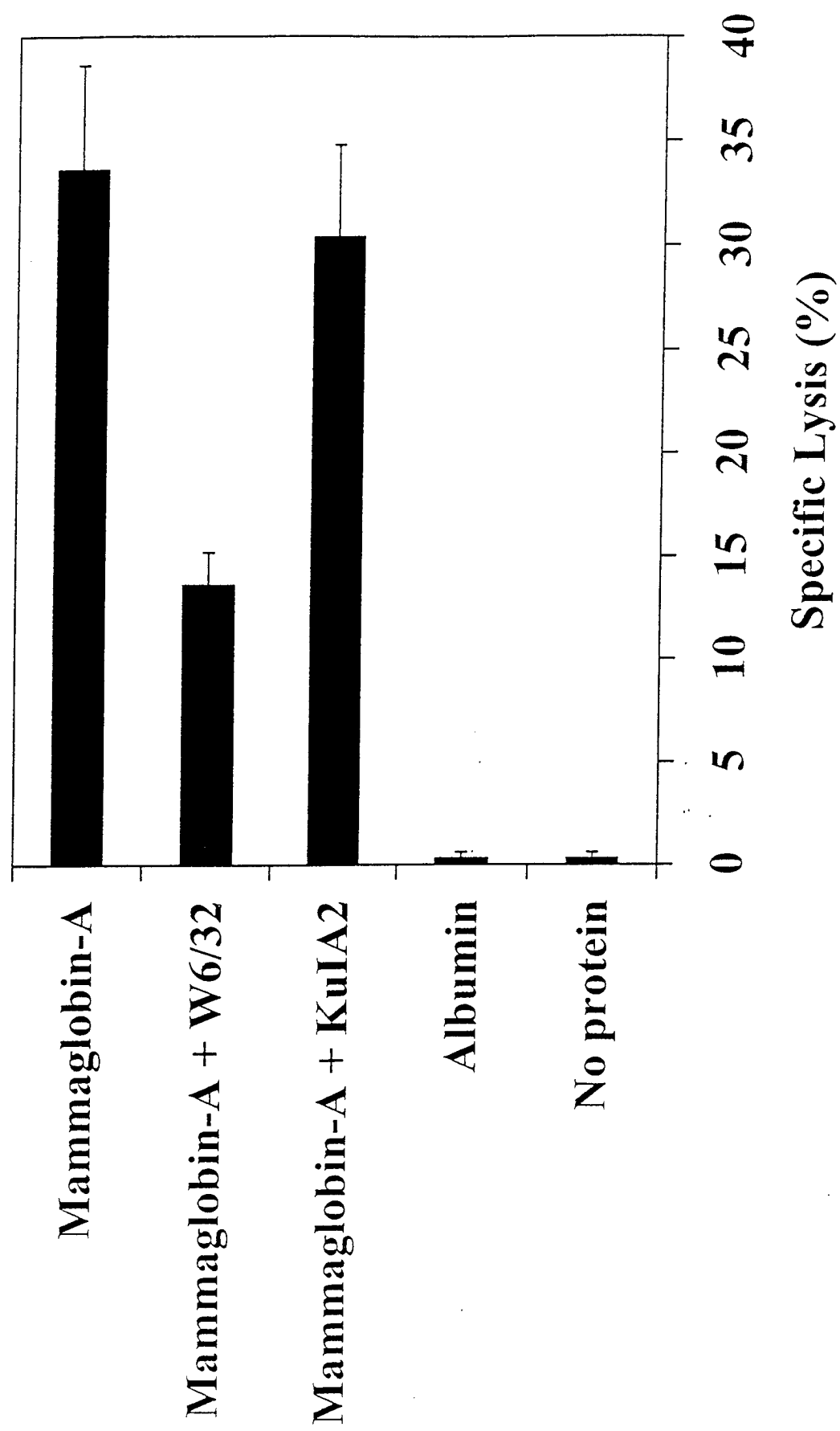
¹HLA typing was performed by oligonucleotide sequence-specific primers that provided low to medium resolution for HLA-A genes (PEL-FREEZ, Brown Deer, WI) and high resolution for HLA-A2 genes (GenoVision Inc., Exton, PA).

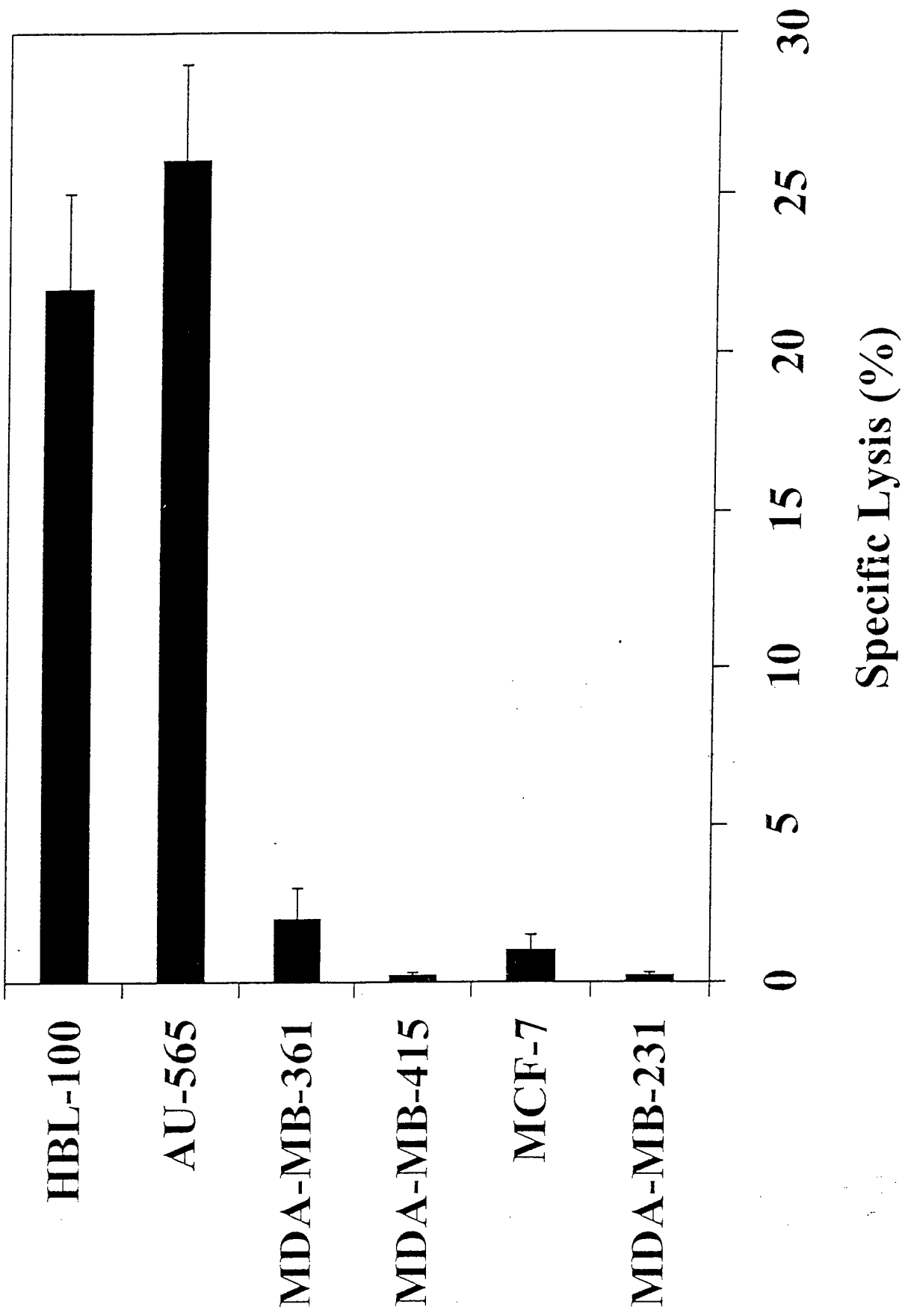
²Mammaglobin-A expression was determined by reverse transcriptase-polymerase chain reaction using sequence-specific primers as previously described (1).

Figure Legends

Figure 1. Mammaglobin-specific cytotoxic activity by CD8+ CTLs generated *in vitro* against recombinant mammaglobin-A. A CD8+ CTL line was generated from an HLA-A2+ breast cancer patient by means of five weekly *in vitro* stimulations with autologous dendritic cells pulsed with mammaglobin-A. The mammaglobin-A-restricted cytotoxic activity of the resulting CD8+ CTL line was evaluated against dendritic cells pulsed with mammaglobin-A or albumin. For antibody blocking analysis, the target cells were incubated with the W6/32 or the KUIA2 monoclonal antibodies for 30 minutes before addition of the effector CD8+ CTLs.

Figure 2. Lysis of breast cancer cells by CD8+ CTLs generated *in vitro* against mammaglobin-A-derived peptides. A CD8+ CTL line was generated from an HLA-A2+ breast cancer patient by means of five weekly *in vitro* stimulations with autologous dendritic cells pulsed with mammaglobin-A. The mammaglobin-A-restricted cytotoxic activity of the resulting CD8+ CTL line was evaluated against six breast cancer cell lines with different profiles of HLA-A2 and mammaglobin-A expression.





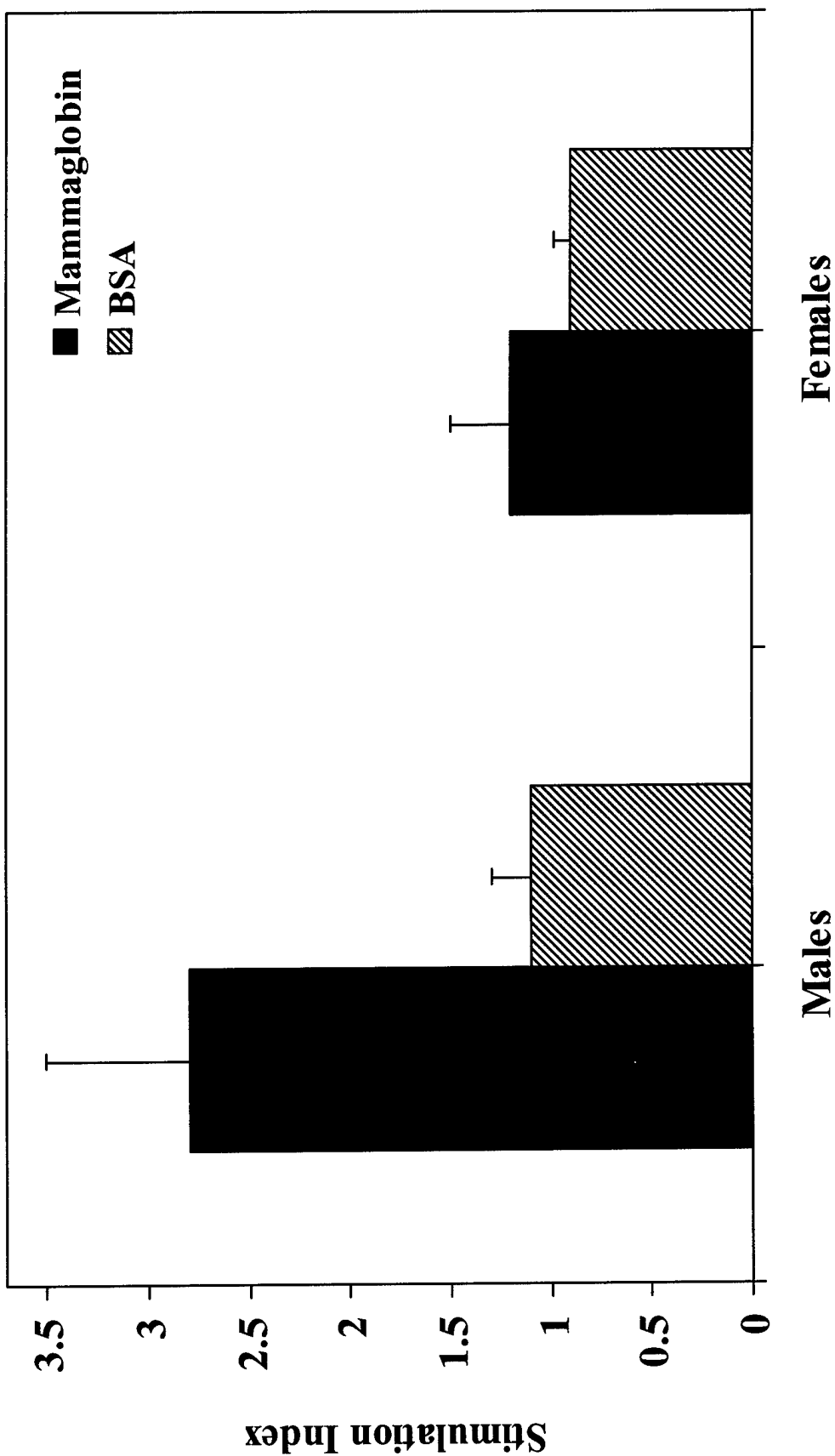


Figure 1. Lack of mammaglobin-specific T cell proliferative responses in healthy female individuals as compared to healthy male individuals.

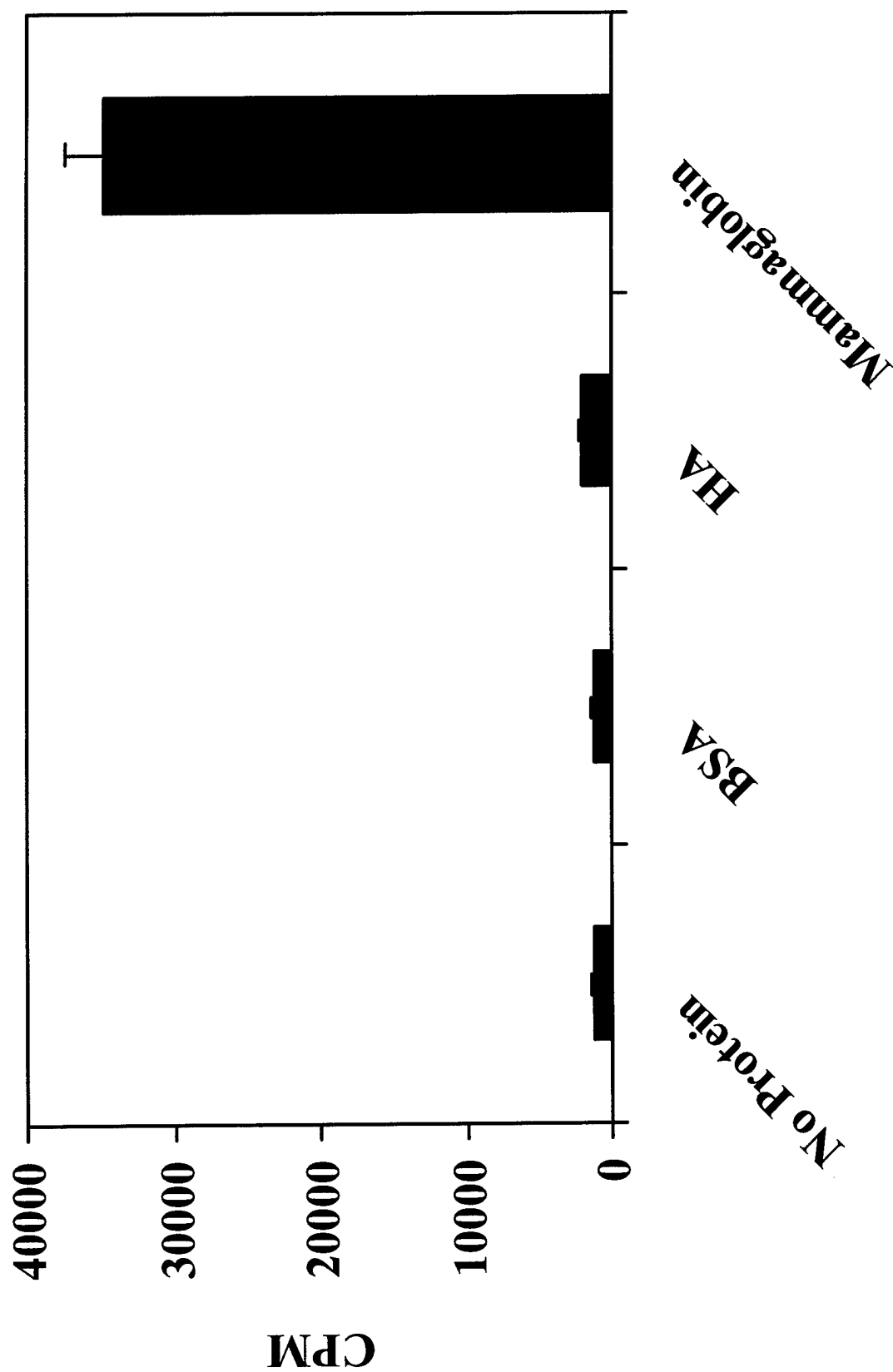


Figure 2. Mammaglobin-specific proliferative response by CD8+ T cells generated *in vitro* against recombinant mammaglobin.

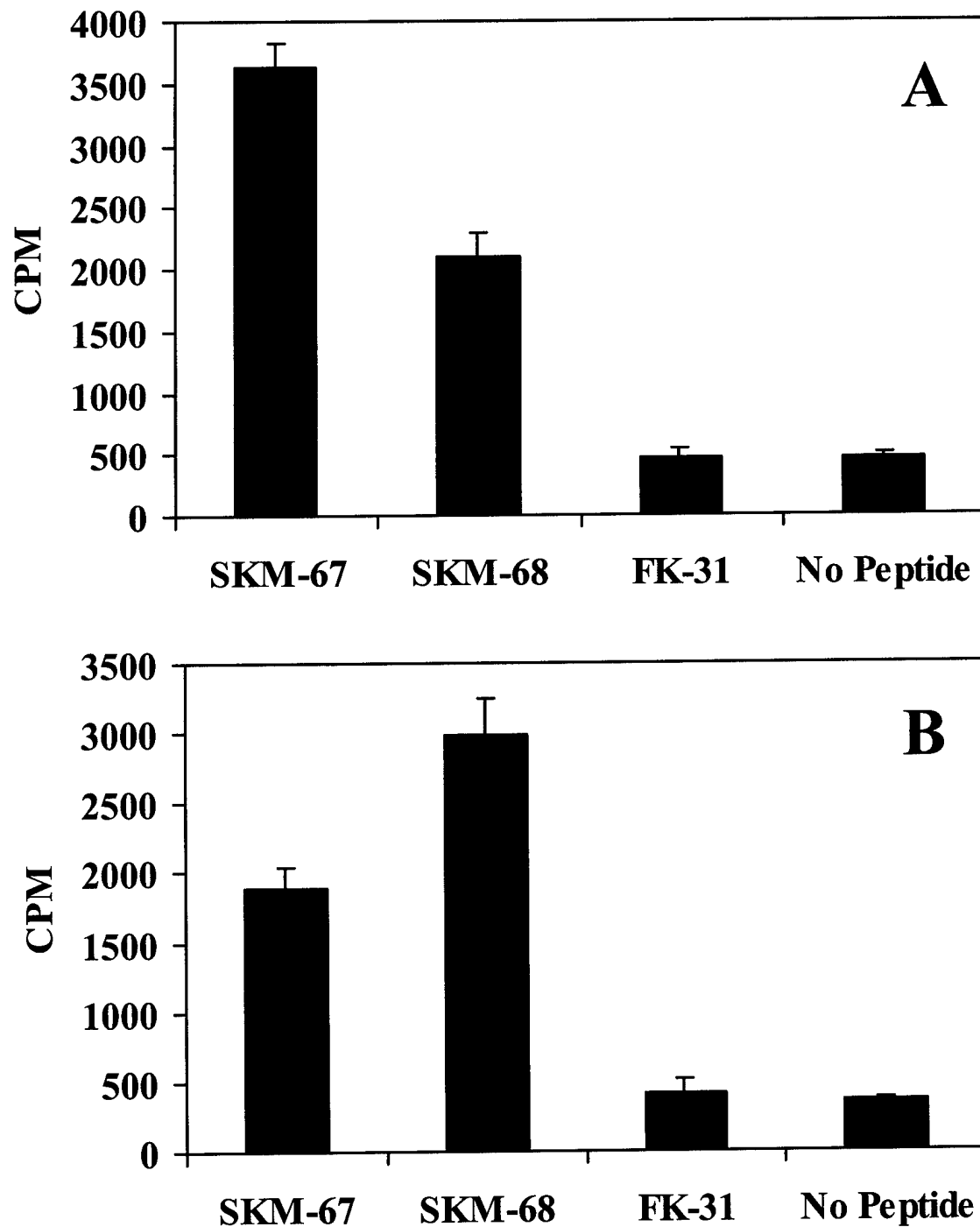


Figure 3. Peptide-specific proliferative response by CD8+ T cells generated *In vitro* against mammaglobin-derived peptides.

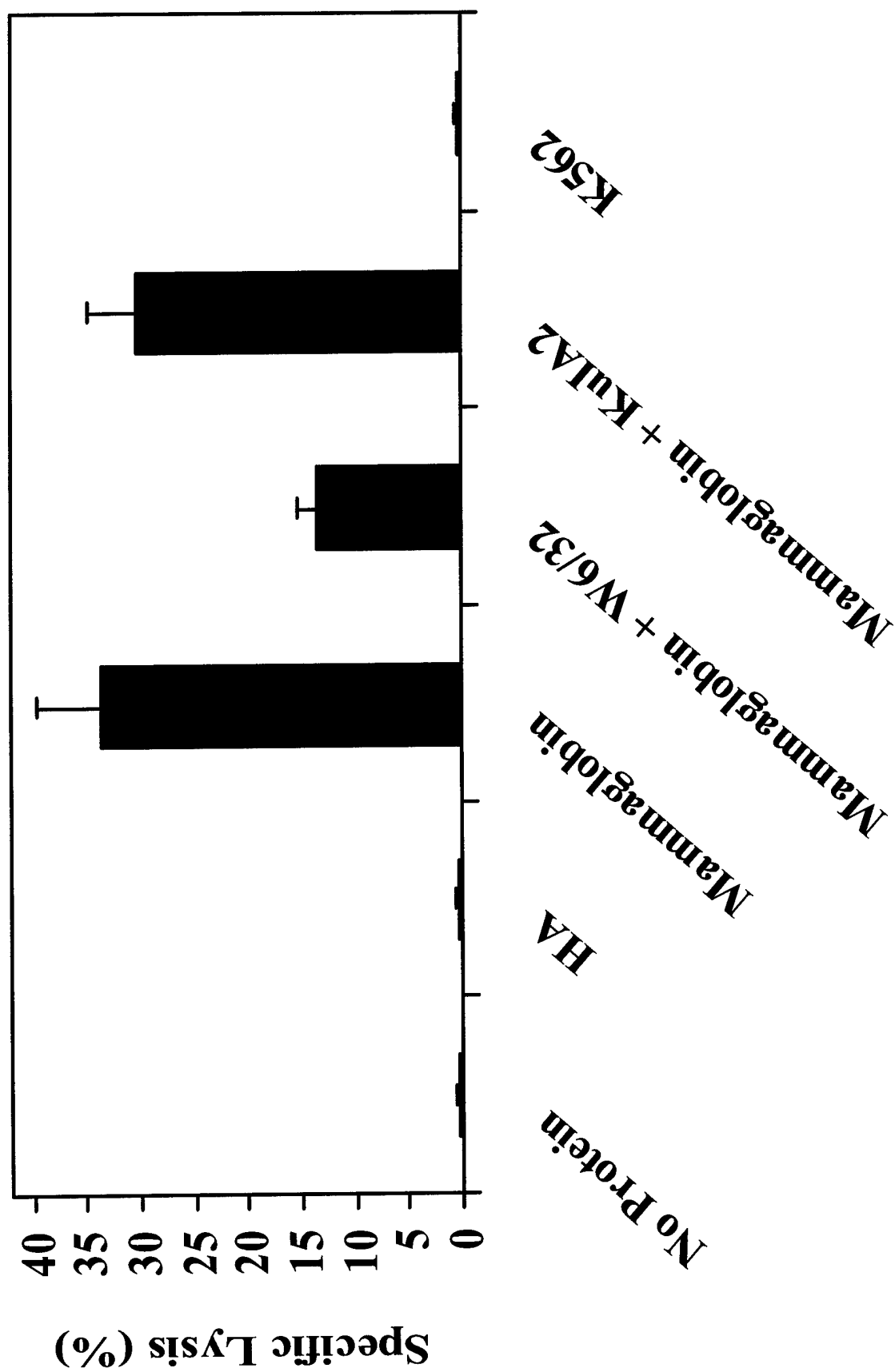


Figure 4. Mammaglobin-specific CTL activity by CD8+ T cells generated *in vitro* against recombinant mammaglobin.

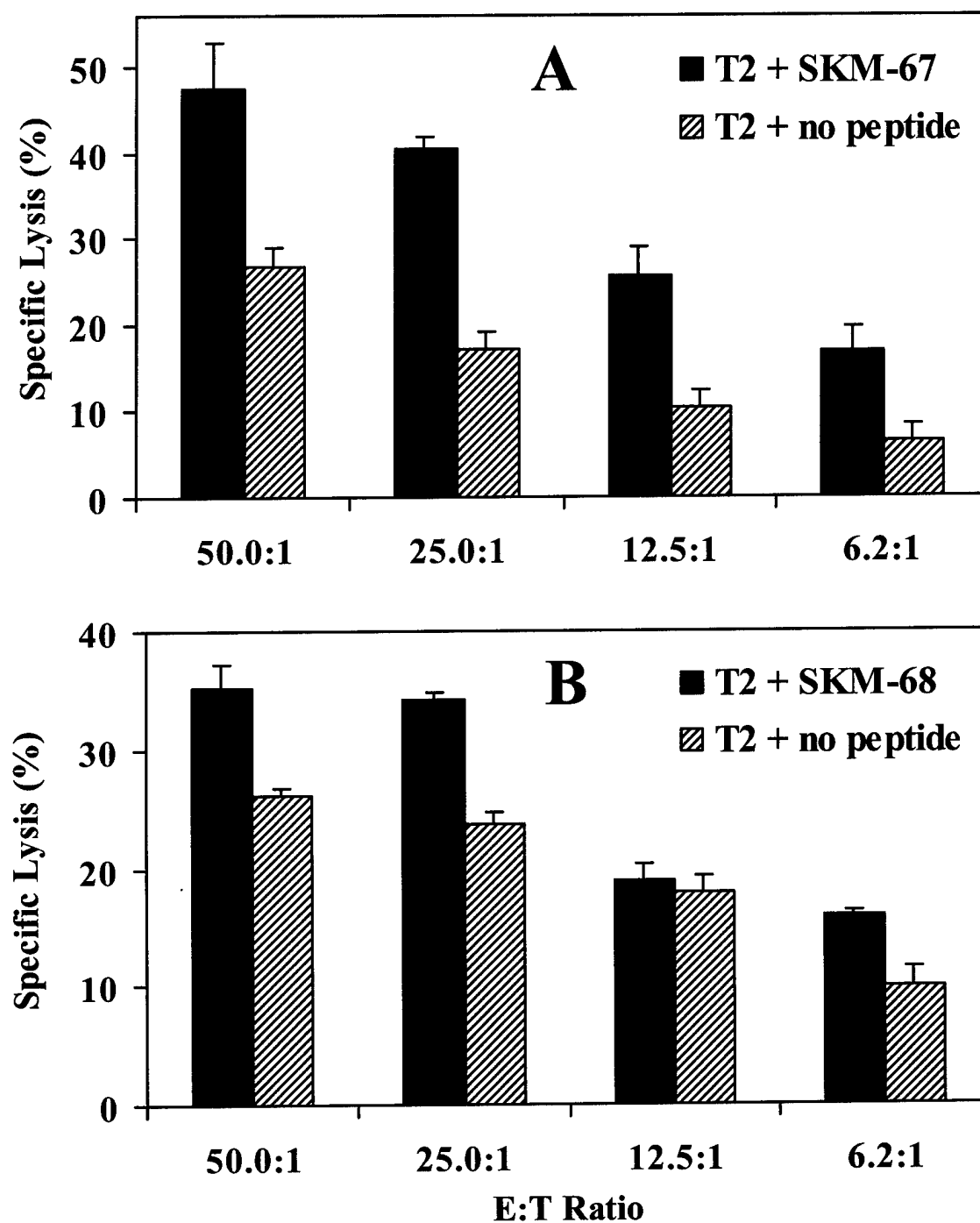


Figure 5. Peptide-specific CTL activity by CD8⁺ T cells generated *in vitro* against mammaglobin-derived peptides.

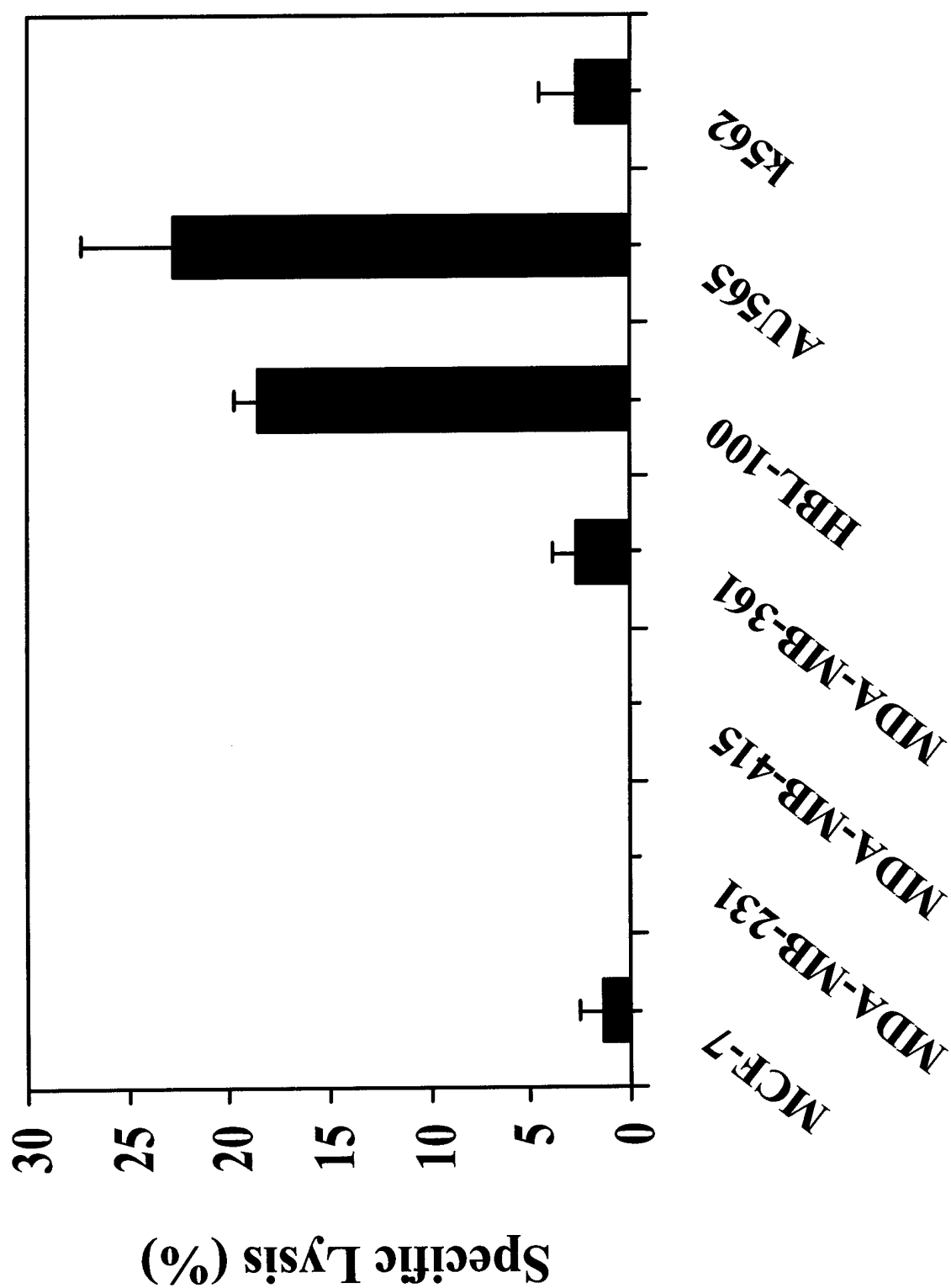
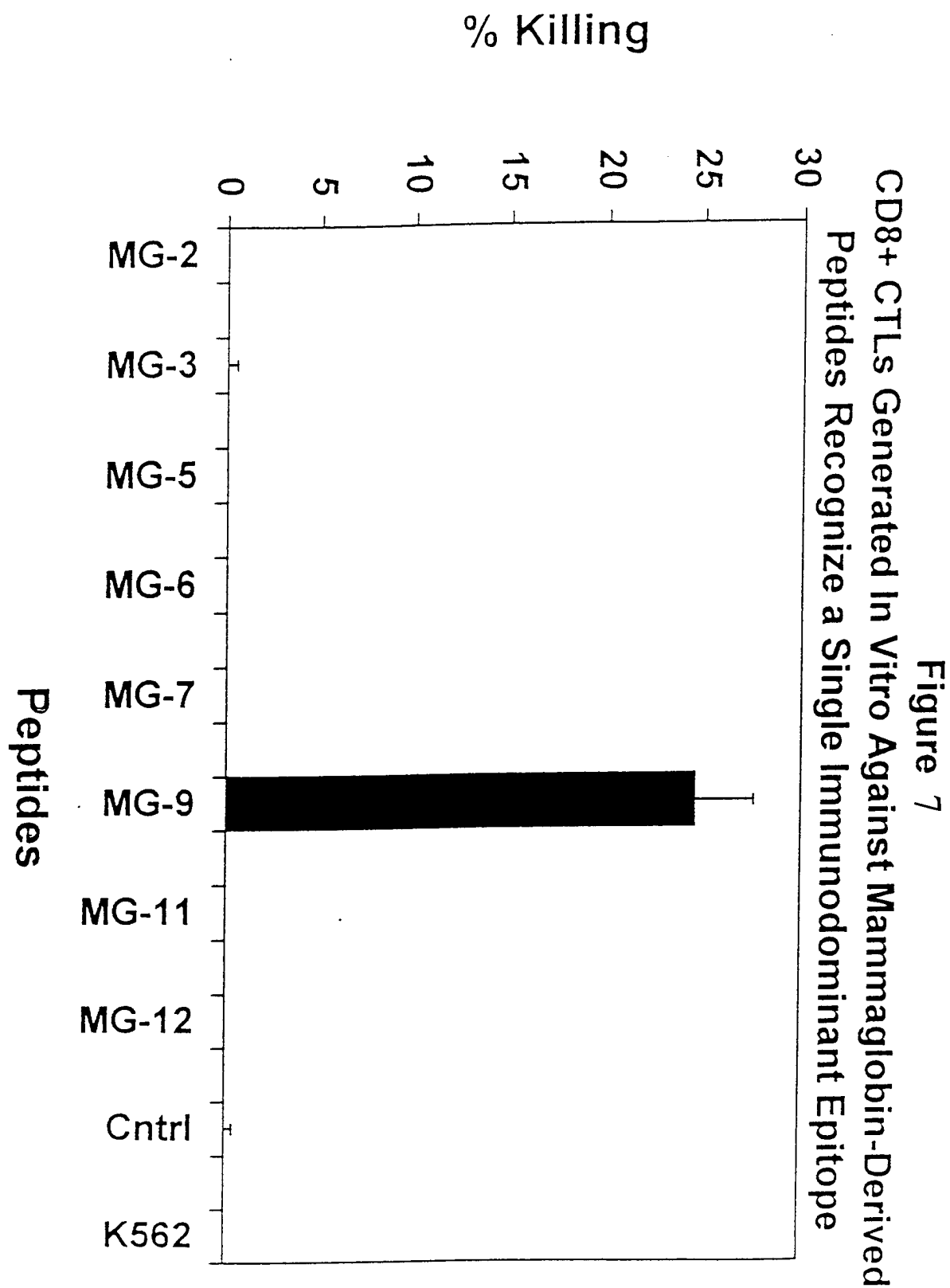
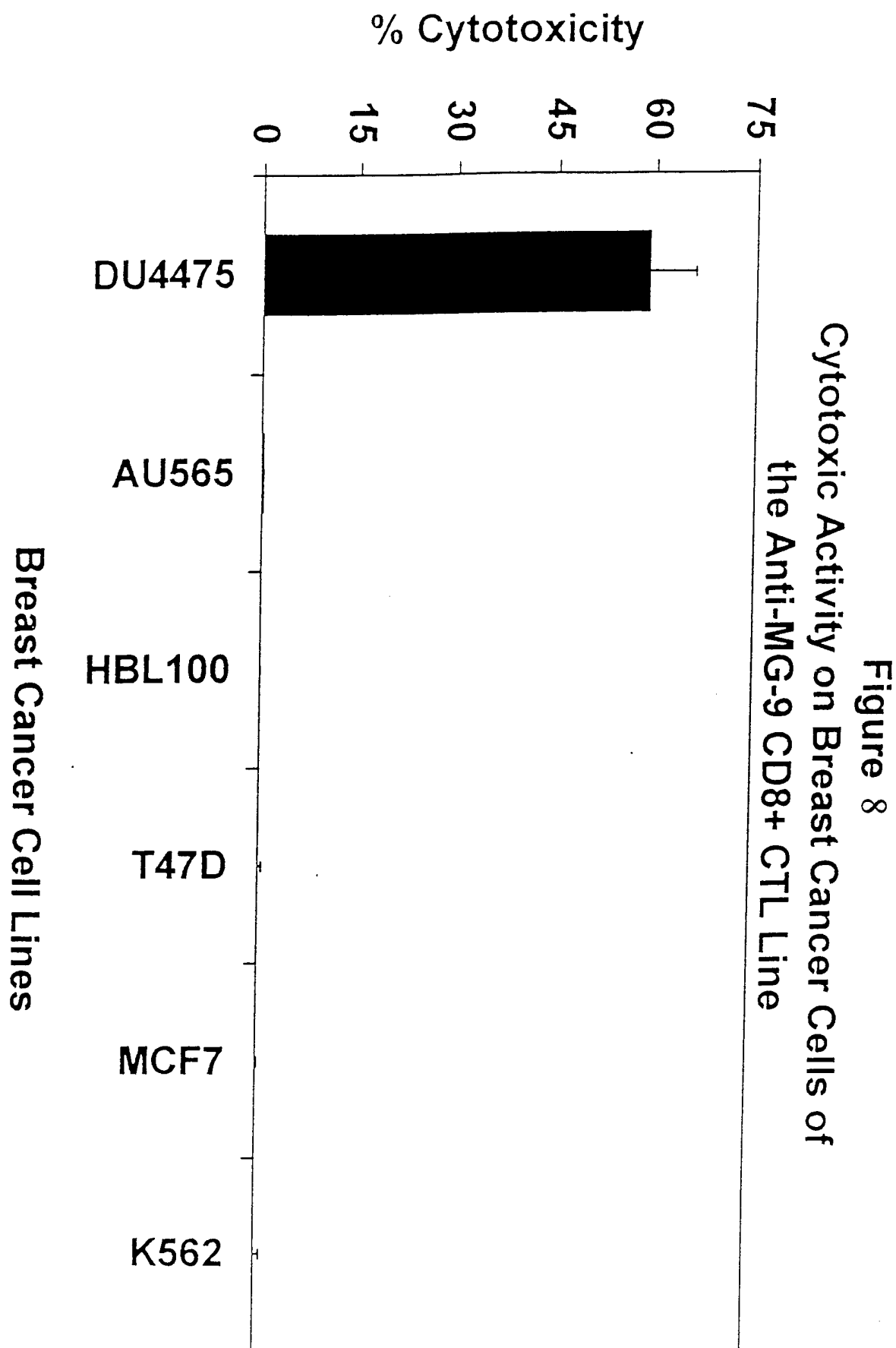


Figure 6. Mammaglobin-specific MHC-restricted CTL activity on breast cancer cells by CD8+ T cells generated *in vitro* against recombinant mammaglobin.





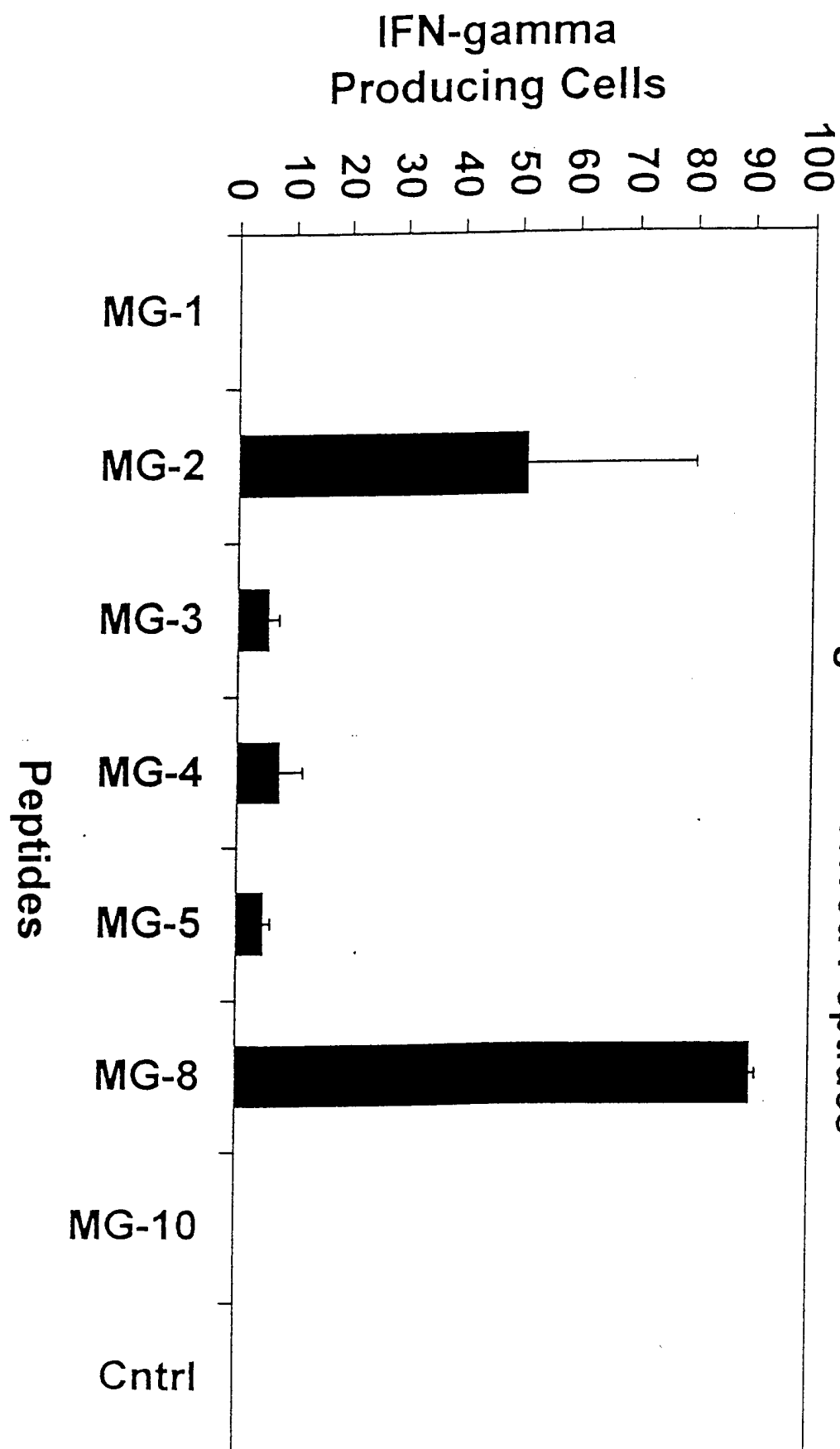


Figure 9
In Vivo Response to HLA-A2-Binding
Mammaglobin-Derived Peptides

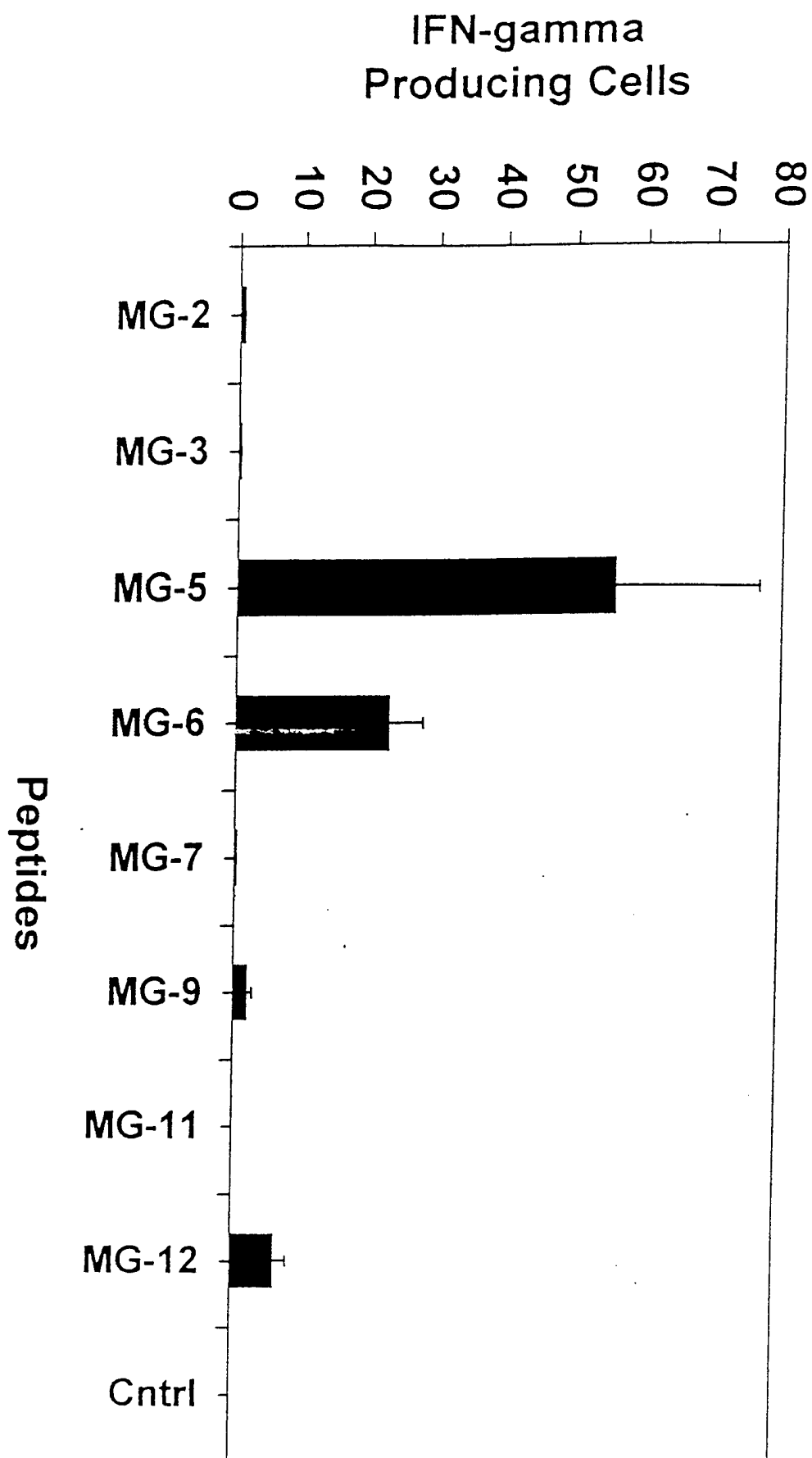


Figure 10
In Vivo Response to HLA-A3-Binding
Mammaglobin-Derived Peptides

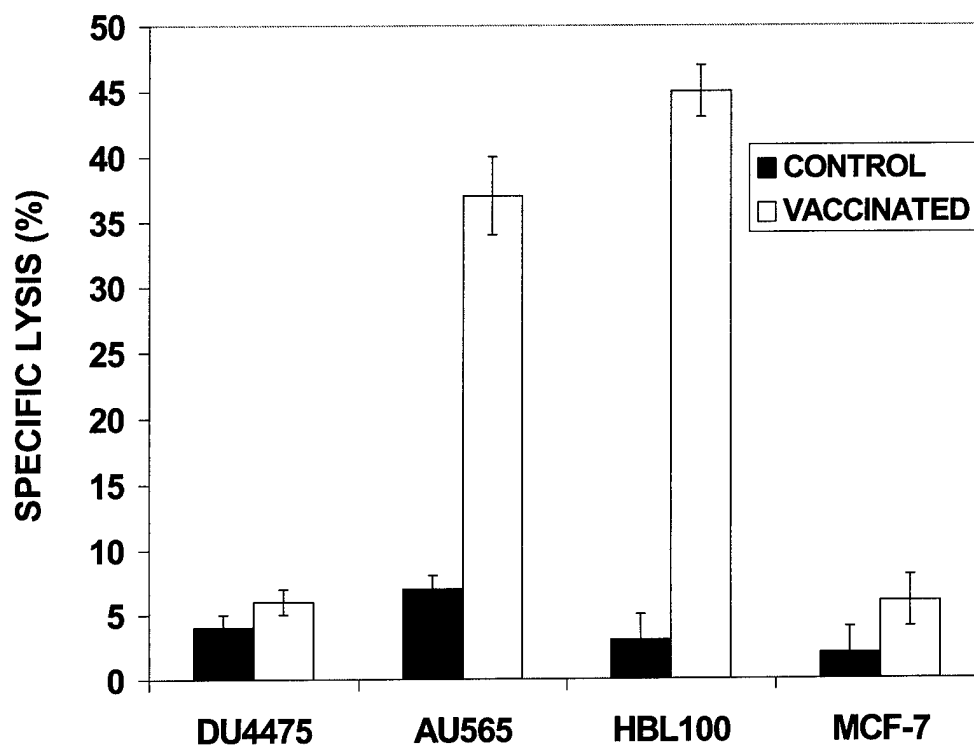


Figure 11: HLA-A2-restricted, mamaglobin-A-specific lysis of breast cancer cells by CD8+ CTLs from HLA-A2+/human CD8+ mice immunized with mamaglobin-A cDNA.

Table 1. Breast cancer cell lines used in this study

Breast Cancer Cell Line	Tumor Classification	HLA-A Phenotype^a	Mammaglobin Expression^b
MCF-7	Adenocarcinoma	HLA-A2,-	-
MDA-MB-231	Adenocarcinoma	HLA-A2, -	-
HBL-100	Adenocarcinoma	HLA-A1,2	+
AU-565	Adenocarcinoma	HLA-A2,3	+
MDA-MB-361	Adenocarcinoma	HLA-A1,24	+
MDA-MB-415	Adenocarcinoma	HLA-A30,33	+

Table 2. Frequency of mammaglobin-reactive T cells in peripheral blood of breast cancer patients and healthy female individuals

Study Subjects	Frequency of Proliferating T cells ^{a,b}	Frequency of CTLs ^{a,c}
Patients		
01	9.00x10 ⁻⁵	1.10x10 ⁻⁵
02	3.31x10 ⁻⁵	5.98x10 ⁻⁵
03	2.74x10 ⁻⁵	1.84x10 ⁻⁵
04	1.07x10 ⁻⁵	3.36x10 ⁻⁵
05	1.88x10 ⁻⁵	4.80x10 ⁻⁶
06	6.73x10 ⁻⁵	2.06x10 ⁻⁵
07	0	2.02x10 ⁻⁵
Mean ± SD:	3.5x10 ⁻⁵ ± 3.2x10 ⁻⁵	2.4x10 ⁻⁵ ± 1.8x10 ⁻⁵
Controls		
01	0	8.00x10 ⁻⁷
02	4.00x10 ⁻⁷	0
03	1.70x10 ⁻⁶	5.00x10 ⁻⁶
04	1.30x10 ⁻⁶	1.50x10 ⁻⁵
05	5.10x10 ⁻⁶	3.60x10 ⁻⁶
06	1.40x10 ⁻⁶	1.10x10 ⁻⁶
07	8.00x10 ⁻⁷	1.90x10 ⁻⁶
Mean ± SD:	1.50x10 ⁻⁶ ± 1.70x10 ⁻⁶	3.90x10 ⁻⁶ ± 5.20x10 ⁻⁶

^aResults are expressed as the reciprocal of the precursor frequency.

^bFrequency of proliferating T cells: Patients vs. Controls, P = 0.03 (two-tailed student's T test).

^cFrequency of CTLs: Patients vs. Controls, P = 0.03 (two-tailed student's T test).

Table 3
Breast Cancer Cell Lines

Breast Cancer Cell Line	Tumor Classification	HLA-A Phenotype	Mammaglobin Expression
DU-4475	Adenocarcinoma	HLA-A3,31	Positive
AU-565	Adenocarcinoma	HLA-A2,3	Positive
HBL-100	Adenocarcinoma	HLA-A1,2	Positive
T-47D	Ductal Carcinoma	HLA-A3,26	Negative
MCF-7	Adenocarcinoma	HLA-A2,-	Negative

**IDENTIFICATION OF HLA-A2-RESTRICTED T
CELL EPITOPES FROM MAMMAGLOBIN-A, A
NEW BREAST CANCER-SPECIFIC ANTIGEN**

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The mammaglobin-A gene encodes a glycoprotein that is highly over-expressed in human breast cancer (BC) cell lines and primary breast tumors. Since the pattern of expression of mammaglobin-A is restricted to mammary epithelium and metastatic breast tumors, the induction of mammaglobin-specific T cell immune responses may provide an important approach for designing specific tumor-targeted immunotherapy. Therefore, the purpose of this study was to identify HLA-A2-restricted mammaglobin-A-derived CD8⁺ T cell epitopes, and to determine whether CD8⁺ cytotoxic T lymphocytes (CTL) developed in vitro against mamaglobin-A-derived peptides have the capacity to kill BC cells naturally expressing mammaglobin-A.

A computer analysis of the mammaglobin-A aminoacid sequence was performed to identify seven mammaglobin-A-derived peptides with the highest binding score to the HLA-A2 molecule (Mam-1-7). By means of ELISPOT analyses we determined that CD8⁺ T cells from BC patients (n=5) react to the Mam-1 (83-92), Mam-2 (2-10), Mam-4 (66-74), and Mam-7 (32-40) peptides and to a lesser extent to the Mam-3 (4-12) peptide. A CD8⁺ T cell line generated in vitro against the pool of mammaglobin-A-derived peptides showed significant HLA-A2-restricted cytotoxic activity against mammaglobin-A-positive but not mammaglobin-A-negative BC cells in vitro.

The results from this study indicate that mammaglobin is a breast cancer-associated antigenic protein. The use of HLA-A2-restricted mammaglobin-A epitopes could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

**IDENTIFICATION OF HLA-A3-RESTRICTED T
CELL EPITOPES FROM MAMMAGLOBIN-A, A
NEW BREAST CANCER-SPECIFIC ANTIGEN**

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The mammaglobin-A gene encodes a glycoprotein that is highly over-expressed in human breast cancer (BC) cell lines and primary breast tumors. Since the pattern of expression of mammaglobin-A is restricted to mammary epithelium and metastatic breast tumors, the induction of mammaglobin-specific T cell immune responses may provide an important approach for designing specific tumor-targeted immunotherapy. Therefore, the purpose of this study was to determine whether mammaglobin-A-reactive CD4+ and CD8+ T cells are activated in vivo in BC patients, to identify HLA-A3-restricted mammaglobin-A-derived CD8+ T cell epitopes, and to determine whether CD8+ cytotoxic T lymphocytes (CTL) developed in vitro against mamaglobin-A-derived peptides have the capacity to kill BC cells naturally expressing mammaglobin-A.

By limiting dilution analysis, we determined that the frequency of mammaglobin-reactive CD4+ and CD8+ T cells in BC patients (n=7) is significantly higher than that observed in healthy female individuals (n=7) ($P = 0.025$ and $P = 0.029$, respectively). A computer analysis of the mammaglobin-A aminoacid sequence was performed to identify eight mammaglobin-A-derived 9-mer peptides with the highest binding score to the HLA-A3 molecule (Mam-1-8). By means of ELISPOT analyses we determined that CD8+ T cells from BC patients (n=5) mainly react to the Mam-3 (2-10) and the Mam-4 (55-63) peptides and to a lesser extent to the Mam1 (23-31) and Mam-8 (58-66) peptides. A CD8+ T cell line generated in vitro against the pool of mammaglobin-A-derived peptides showed significant cytotoxic activity only to HLA-A3+ cells loaded with the Mam-1 peptide. This CD8+ T cell line displayed an HLA-A3-restricted cytotoxic activity against mammaglobin-A-positive but not mammaglobin-A-negative BC cells in vitro.

The results from this study indicate that mammaglobin is a breast cancer-associated antigenic protein. The use of HLA-A3-restricted mammaglobin-derived epitopes could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

CYTOTOXICITY OF BREAST CANCER CELLS MEDIATED BY HUMAN DENDRITIC CELLS

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Dendritic cells (DC) are an important subset of antigen presenting cells characterized by their potent capacity to activate immunologically naïve T cells. However, their role in effector function in tumor resistance is less well characterized. The immune surveillance role of DC has been documented in several systems, which involves the secretion of important immunoregulators such as TNF-alpha. DCs have been found to infiltrate to the areas surrounding human solid tumors and the density of their infiltration has been directly correlated with the condition and prognosis of the disease. Besides the classical antigen presenting role of DC to T cells, direct role of DC against tumor has been reported in recent times.

In this study, we analyzed the interaction between human DC and a several breast cancer cell lines in vitro. DCs showed variable levels of cytotoxicity as well as growth inhibition of breast cancer cell lines. We report here that activated DCs acquire a potent anti-tumor effect against breast cancer cell lines leading to growth inhibition and apoptosis of the tumor cells. The anti-tumor effect of DC was augmented by pro-inflammatory stimuli induced by lipopolysaccharide (LPS) treatment. TNF-alpha produced after DC activation was responsible for the anti-tumor activity of DC. IFN-gamma, IL-15, or LPS treatment of DC markedly augmented the effector function of DC against most of the breast cancer cell lines, indicating heterogeneity of susceptibility to cytokine-mediated damage. Treatment of activated DCs or cell-free supernatants with a neutralizing anti-TNF-alpha antibody significantly reduced the DC anti-tumor activity against the breast cancer cells. These results suggest that in addition to their predominant role as immune regulatory cells, DCs could serves as an innate effector cell in tumor immunity. This anti-tumor effect of DCs is associated with induction of apoptosis and provides a new mechanism by which DCs mediate anti-tumor function in breast cancer.

**INDUCTION OF CD8+ T CELL CYTOTOXICITY TO
HUMAN BREAST CARCINOMA BY DNA
VACCINATION WITH THE MAMMAGLOBIN-A
GENE IN HLA-A2+ MICE**

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Genetic immunization against tumor antigens is an effective way to induce an immune response to prevent cancer progression. The mammaglobin-A gene encodes a glycoprotein that is over-expressed in human breast cancer cell lines and primary breast tumors. Given the high frequency of expression in breast cancer cells, its relative low expression in normal breast tissue and its total lack of expression in other normal tissues, this novel protein could be targeted for the development of an effective immunomodulatory therapy against breast cancer.

In this study, we constructed a DNA expression vector encoding the full-length mammaglobin-A cDNA (PCI.Neo-MamA) and evaluated the efficacy of intramuscular injection of this plasmid to induce breast cancer-specific immune response in HLA-A2/human CD8 double-transgenic mice. Spleen cells obtained from mice immunized with this plasmid were analyzed for their ability to recognize mammaglobin-A-derived peptides and kill breast cancer cell lines. A computer analysis of the mammaglobin-A amino acid sequence was performed to identify eight mammaglobin-A-derived 9-mer peptides with the highest binding score to the HLA-A2 molecule. By means of ELISPOT analysis using the Tap-deficient T2 cell line loaded with individual peptides, we determined that spleen cells from immunized mice recognized the Mam-1 (amino acids 83-92) and Mam-2 (amino acids 2-10) peptides and to a lesser extent the Mam-4 (amino acids 66-74) and Mam-6 (amino acids 80-89) peptides. This observation was further substantiated by the ⁵¹Cr-release cytotoxicity assays, wherein there was specific lysis of T2 cells loaded with peptides Mam-1 and Mam-2. Further, spleen cells from immunized mice showed significant HLA-A2-restricted cytotoxic activity against mammaglobin-A-positive but not mammaglobin-A-negative breast cancer cell lines.

The results from this study suggest that mammaglobin cDNA vaccination could provide a novel approach for designing an effective immunotherapy against breast cancer.
